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(57) Abstract

The invention relates to cataractogenesis relating to disruption of an endogenous α3 connexin gene and the degradation of lens crystallin proteins, particularly γ–crystallin proteins. More specifically, the invention describes the production of a non–human mammal in which the expression of α3 connexin protein is disrupted via the insertion of an exogenous disrupted α3 connexin gene into the genomic DNA of a non–human mammal thereby producing a non–human lacking functional α3 connexin protein or α3 connexin protein, the absence of which leads to age–related cataract formation. The invention further relates to compositions and methods of making and use for diagnostic and therapeutic applications relating to cataracts.

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CATARACTOGENESIS AND DISRUPTION OF D3 CONNEXIN GENE IN MAMMALS AND METHODS OF USE

Technical Field

The invention relates to cataratogenesis and the disruption of α3 connexin gene. More specifically, the invention concerns the insertion of an exogenous disrupted α3 connexin gene into the genomic DNA of a non-human mammals resulting in the lack of α3 connexin protein, the absence of which leads to age-related cataract formation. The invention further relates to vector constructs containing the disrupted α3 connexin gene. Methods of making and using the α3 disrupted connexin gene for preparing mammals containing mutant α3 alleles that result in the absence of functional α3 connexin protein are described. Screening methods are also described using such mammals to study cataract biology. The invention also relates to *in vitro* and *in vivo* screening methods to identify compounds useful in preventing or treating cataracts.

This invention further relates generally to the prevention of cataract formation or the treatment of existing cataracts with interleukin-1β converting enzyme (ICE) inhibitors.

Background of the Invention

The vertebrate lens consists of a single anterior surface layer of cuboidal epithelial cells covering the bulk of the lens, formed by lens fiber cells. The maintenance of the metabolic activities of these interior cells has been suggested to depend on a unique network for current flow, in the form of ions, that connects interior mature fibers, peripheral newly-differentiating fibers and anterior epithelia cells (Mathias et al., *Physiol. Rev.*, 77:21-49 (1997); Robinson and Patterson,

Curr. Eye Res., 2:843-847 (1983)). Gap junction channels formed between fiber-fiber, fiber-epithelial and epithelial-epithelial cells have been proposed to be a major part of this network, and to play a important role in lens homeostasis (Goodenough, Sem. in Cell Biol., 3:49-58 (1992); Rae et al., J. Mem. Biol., 150:89-103 (1996)).

Gap junction channels consist of two hemichannels, called connexons, that are located in the plasma membranes of two adjacent cells. Each connexon is composed of 6 subunits of the protein termed connexin. These channels, commonly found in arrays called plaques, allow small molecules, of molecular weight less than 1000 daltons, to pass directly between the cytoplasms of the two connected cells. Thus far, more than a dozen connexin genes have been identified in vertebrates (Bruzzone et al., Eur. J. Biochem., 238:1-27 (1996); Kumar and Gilula, Cell, 84:381-388 (1996)). Three connexin genes have been reported to be expressed in the lens. The α_1 connexin (Cx43) has been detected in mouse lens epithelial cells (Yancey et al., Devel., 114:203-212 (1992)). Both α3 (Cx46) and α8 (Cx50) connexin have been detected in the elongating primary fibers, as well as in the secondary fibers during lens development, and they are colocalized to the same gap junction plaques (Paul et al., J. Cell Biol., 115:1077-1089 (1991)).

The most abundant proteins in the vertebrate lens are crystallins, which account for more than 90% of the lens soluble proteins (Bloemendal, New York, John Wiley and Sons, pp 85-136 (1981); Harding and Crabbe, In: The Eye, Davson, H. (ed) Academic Press, pp 207-492 (1984). Crystallins from mammalian lenses are divided into three major classes: α-, β- and γ-crystallins. Lens transparency results from the suprastructural organization of the lens that is created by both plasma membrane components and cytoplasmic proteins, including the crystallins.

However, the molecular mechanisms by which this organization is generated and maintained are not well understood.

Although connexin proteins have been suggested to play a critical role in vertebrate lens development and in maintaining lens transparency, the exact nature of their structural and functional roles have not been identified.

Brief Description of the Invention

The present invention now provides an *in vivo* animal model system and compositions to produce such for studying the role of α3 connexin protein in lens development and for identifying drugs useful in the treatment of cataracts that develop postnatally in α3-connexin-deficient mammals.

In one aspect, the invention provides a non-human mammal, preferably a mouse, and its progeny either containing a nonfunctional α3 connexin protein or lacking functional α3 connexin protein altogether. The resultant non-human mammal is referred to as a α3 connexin-deficient non-human mammal. The α3 connexin deficiency is generated by the insertion of a nucleic acid sequence comprising at least a portion of the α3 connexin gene into the genome of the mammal. The inserted α3 connexin nucleic acid sequence present in the genome is referred to as a disrupted α3 connexin gene. In preferred embodiments, a disrupted α3 connexin gene lacks at least one transmembrane-encoding region and more preferably two regions and most preferably four regions. In a further embodiment, an α3 connexin gene lacks one or more nucleotides in the protein coding region, the presence of which results in a nonfunctional α3 connexin protein. In another embodiment, an α3 connexin gene contains one or more nucleotide substitutions, the presence of which also results in a nonfunctional α3 connexin protein.

In another aspect, the invention provides a nucleic acid

sequence corresponding to the above-described disrupted α3 connexin gene operably linked to a plasmid vector. A preferred plasmid comprises a nucleic acid sequence that is an α3 connexin gene lacking four transmembrane domains. An α3 connexin gene-containing plasmid further comprises at least a selectable marker, preferably the gene encoding neomycin resistance. In other embodiments, an α3 connexin gene-containing plasmid also comprises a marker gene for use in detecting the presence of an α3 mutant allele in a mammal of this invention. A preferred marker gene encodes lacZ. A preferred disrupted α3 connexin gene-containing plasmid is the plasmid, designated pα3-GZK, on deposit with American Type Culture Collection having ATCC Accession Number 97848, the deposit of which is further described below. In further aspects, the invention provides an embryonic stem cell, preferably a JS-1 or an ES cell, containing a disrupted α3 connexin gene.

In another aspect, the invention provides methods for making a disrupted α3 connexin gene and plasmids containing such genes.

In a further aspect, the invention provides a method for studying α3-dependent nuclear fiber cataract formation in the lens of the mammalian model system as a basis for human cataract formation.

In yet another aspect, the invention provides a method of using the α3 connexin-deficient mammal that develop postnatal nuclear lens opacities of this invention for identifying compounds for therapeutic treatment thereof comprising administering the compounds to a mammal and determining the effects of the compounds. The lens is either left in place in the mammal or is explanted into culture, i.e., an *in vitro* culture. For preventing cataracts, the test compound is applied before the cataracts form in the model. The mammal or cultured

lens is then observed over a preselected period of time to assess the effectiveness of the test compound on preventing the formation of cataracts. Similarly, compounds are tested in the same system for the effect on delaying the progression of cataract formation, for stopping the progression and also for reversing cataract formation.

The methods of the present invention are further based on the related discovery of an interleukin-1β converting enzyme (ICE) or functional analog thereof in the effected animal that resulted as a consequence of the gene disruption. Thus, in a therapeutic aspect, the invention provides a method for preventing cataract formation or treating a formed cataract in a subject by the administration of a therapeutically effective cataract inhibiting amount of an interleukin-1β converting enzyme (ICE) inhibitor tc a subject. Treating a formed cataract includes delaying, stopping or reversing the progression of cataract formation. Preferred ICE inhibitors are delivered topically, orally or intravenously. Acceptable carriers for mediating such delivery are also contemplated.

Methods are contemplated for screening candidate compounds to identify new inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog. One preferred approach is based on the known cleavage pattern of a crystallin lens protein, preferably γ, by ICE or functional ICE analog. An inhibitor of ICE or an analog is then identified by detecting an alteration in the cleavage pattern of the lens protein. In other words, an inhibitor is identified by detecting an uncleaved substrate that is normally cleaved in the absence of an inhibitor.

Another preferred screening assay for an inhibitor is to use any substrate of ICE including a crystallin lens protein, preferably γ, in conjunction with a homogenate of lens isolated from a non-human mammal having disrupted alleles of α3 connexin

gene, where the homogenate contains ICE or functional ICE analog. As before, an inhibitor is identified by detecting an uncleaved substrate that is normally cleaved in the absence of an inhibitor.

For either screening method, the substrate may either be in a mixture with other proteins or may be in purified form. A preferred ICE substrate is γ-crystallin lens proteins. The assay is preferably conducted where the lens protein, ICE or functional ICE analog, and the candidate inhibitor are in solution.

The present invention also contemplates kits and diagnostic systems with the system and compositions as described above to identify inhibitors of ICE or functional analog thereof as well as to identify compounds useful in preventing, treating or reversing the formation of cataracts.

Other variations and uses of the present invention will be apparent to one skilled in the art in light of the present disclosures.

Brief Description of Drawings

In the figures forming a portion of this disclosure:

Figure 1A shows the physical maps of the wild type α3 connexin allele, α3 targeting vector, and mutant α3 allele. Restriction enzyme sites are marked as follows: A, Apa I; B, Bam HI; H, Hgi AI; H3, Hind III; and RI, EcoRI. (RI), (A) and (H3) designate the mutated sites after construction. The non-italicized amino acid sequence is the N-terminal 17 amino acid residues of α3 connexin. The amino acid sequence in italics represents the residues encoded by a linker sequence in front of the lac Z gene.

Figure 1B is a Southern blot analysis of an α3 (+/+) ES cell clone and an α3 (+/-) ES cell clone. DNA from ES cells was

digested with Bam HI and hybridized with the probe A (shown in Figure 1A). The 12 kb band corresponds to the wild-type α3 allele, and the 5.5 kb band to the mutant α3 allele.

Figure 1C is a Southern blot analysis of F2 mice. An α3 (+/+), an α3 (+/-), and an α3 (-/-) mouse were analyzed using probe A as used for the ES cell analysis in B.

Figure 1D shows the genotypes of the α3 'knockout' mice that were determined also by PCR, using three primers termed P1, P2 and P3. A 350 bp PCR fragment was generated from the wild-type α3 allele, the size predicted to result from the P1 and P2 primers, and a 500 bp fragment from the mutant α3 allele was generated as predicted from using P1 and P3 primers.

Figure 1E shows total RNAs that were isolated from α3 (+/+), α3 (+/-) and α3 (-/-) lenses from one month old mice, and analyzed by Northern blot using a cDNA probe covering the encoding DNA sequence for the four transmembrane domains of α3 connexin. Fifteen μg RNA was loaded in each lane. The equal intensity of the rRNAs confirms that each lane has an equivalent amount of RNA.

Figure 2 shows the expression pattern of the *lacZ-nls* gene and lens development.

In Figure 2A, three embryos at stage 14 dpc were stained for lacZ as whole mounts. LacZ staining was evident in the lenses from both α3 (+/-) (right) and α3 (-/-) (middle) embryos. The lens of the α3 (+/+) embryo was not detectably stained (left). The α3 genotype of these embryos was detemined by Southern analysis of genomic DNA isolated from the yolk sac.

In Figure 2B, a section along the equatorial plane of an eye from an α3 (-/-) embryo at stage 14 dpc, after whole mount lacZ staining, contains blue stained lens, surrounded by a circle of retinal pigmented epithelium.

Figure 3C shows a frozen section, along the anterior-

posterior axis from the eye of a 2 week old α3 (-/-) mouse, was stained for *lacZ-nls* activity. The nuclei of the newly formed fiber cells contained a strong blue staining, which gradually disappeared towards the interior fibers. A weak blue staining was also observed in the nuclei of the lens epithelial cells located at the anterior surface of the lens (indicated by arrows).

Figure 2D is a histological section of the eye from an α3 (+/+) embryo at stage 18.5 dpc.

Figure 2E is a histological section of the eye from an α3 (-/-) embryo at stage 18.5 dpc.

Figure 3 illustrates the nuclear cataract phenotype of α3 (-/-) mice.

Figure 3A contains the lenses (unfixed) dissected from fresh eyes of α3 (+/+) mice and α3 (-/-) mice at the age of two months, and photographed from their anterior surface with a dissection microscope.

Figure 3B contains a side view (anterior side of the lens on the right and posterior side on the left) of similar lenses embedded in LR white resin after fixation with 2.5% glutaraldehyde. The black staining on the lens surface is the retinal pigmented epithelial cells attached to the lenses. Nuc represents a 2X enlargement of the nuclear region of the cataract lens. A large aggregate associated with the cataract is indicated by an arrow in upper right panel. The scale bar is 1 mm.

Figure 4 illustrates the histological and immunocytochemical analysis of adult mice.

Figure 4A are histological sections from the anterior bow region (left) and the nuclear region (right) of the same cataract lens embedded in LR white resin shown in Figure 3B. The arrow denotes an aggregate of undefined composition in the

nuclear region. The sections were stained with methylene blue. The scale bar is 10 μm.

Figure 4B shows indirect immunofluoresence images with double labeling of α3 (FITC, green) and α8 (RITC, red) connexins in the lens cortex of a frozen section from a one month old α3 (+/+) mouse viewed with laser scanning confocal microscopy. The middle panel is the merged image from the right and left panels; the yellow denotes the co-localization of α3 and α8 connexins.

In Figure 4C, the left and right panels contain the double labeling pattern of α3 and α8 connexins in the lens cortex of a frozen section from an α3 (-/-) mouse at the age of 1 month. No α3 connexin was detectable by α3 antibodies (left panel). However, the large gap junction plaques formed by α8 connexin were still detected by the α8 monoclonal antibodies. Scale bar for images in (B) and (C) is 10 μm.

Figure 5 are freeze-fracture replicas of the lenses of α3 (+/+) and α3 (-/-) mice at the age of 3 weeks.

Figures 5A and 5B contain gap junction plaques (arrows) that were observed in the superficial cortex of the wild-type lens (+/+) and the α3 (-/-) lens.

Figure 5C shows arrays of furrowed membrane fingers in the nuclear region from an α3 (+/+) lens, whereas Figure 5D shows an altered membrane with irregular furrowed structures from the same nuclear region of an α3 (-/-) lens.

Figure 5E shows the square-array membrane particles (5-6 nm size, arrowhead) that were observed as small islands in the furrows of the membrane arrays from the α3 (+/+) lens nucleus, with many interdigitated 9- to 11-nm particles (arrows).

Figure 5F shows that much more extensive areas of the square-array particles (arrowheads) were observed in the disorganized furrowed membranes from the nucleus of α3 (-/-) lenses. The scale bar is 200 nm in all images.

Figure 6 shows lens weight and crystallin profiles.

Figure 6A is a comparison of the total lens weight of α3 (+/+) and α3 (-/-) mice as a function of age. Lenses were weighed immediately after dissection from the eyes. The lens weight in the figure represents an average of lenses from 6 to 16 mice. The filled circles and open triangles represent the total lens weight of α3 (-/-) and α3 (+/+) lenses, respectively.

Figure 6B shows the elution profiles of the total water soluble crystallins from the lenses of α3 (+/+) [Δ] and α3 (-/-) [○] mice at the age of 2 months. α, β, and γ peaks correspond to the three major classes of crystallins.

Figure 6C shows the elution profiles of the total water soluble crystallins from the lenses of α3 (+/+) and α3 (-/-) mice at the age of 4 months.

Figure 6D shows the elution profiles of the total water soluble crystallins from the cortical regions of the lenses of α3 (+/+) and α3 (-/-) mice at the age of 4 months.

Figure 6E shows the elution profiles of the total water soluble crystallins from the nuclear regions of the lenses of α3 (+/+) and α3 (-/-) mice at the age of 4 months. The peak labelled H represents the water-soluble high-molecular weight protein aggregates.

Figure 7 illustrates the electrophoretic characterization of lens NaOH insoluble proteins and crystallins.

In Figure 7A, a Coomassie blue stained gel is shown of the NaOH-insoluble proteins from the lenses of α3 (+/+) (lane 1 and 2) , α3 (+/-) (lane 3 and 4) and α3 (-/-) (lane 5 and 6) littermates at the age of 2 months. Both lenses from a single mouse were used to prepare the NaOH-insoluble proteins; these proteins were divided into two equal portions, which were dissolved in an equal volume of sample buffer, with and without 10 mM DTT. Equal volumes of the samples were

separated in a 12.5% polyacrylamide gel and stained with Coomassie Blue. The samples with 10 mM DTT were in lanes 1, 3 and 5, and those without DTT in lanes 2, 4 and 6. Significant amounts of protein from 17 to 27 kDa in size were observed only in lane 5 which contained the NaOH-insoluble protein from α3 (-/-) lenses in DTT containing sample buffer. Although lane 6 had an equal amount of protein as lane 5, due to the absence of DTT, most of the insoluble protein existed as high molecular weight aggregates which could not enter the 5% stacking gel. The major band, observed in lanes 1-4, corresponds to the major lens membrane protein, MP26. Two arrows on the right side denote the bands (in lane 5) corresponding to the 9 and 11 kDa cleaved γ-crystallins which were purified for determination of their N-terminal peptide sequence.

Figure 7B shows the comparison of crystallin protein profiles in the lenses of α3 (-/-) and α3 (+/+) mice by Western blot analysis. Protein extracts from α3 (+/+) lenses (lanes 1-4) or α3 (-/-) lenses (lanes 5-8) were subjected to SDS-PAGE and Western blotting. All samples were analysed in sample buffer containing 10 mM DTT. Lanes 1 and 5 contained the total lens cortical protein; lanes 2 and 6 contained the water-soluble fraction from the lens nucleus; lane 3 and 7 contained the NaOH-soluble fraction from the lens nucleus; and lanes 4 and 8 contained the NaOH-insoluble fraction from the lens nucleus. Four different polyclonal crystallin antibodies, anti-αA, anti-αB, anti-βB2 and anti-γ, were used to analyze these samples. The position of the intact form of each crystallin protein is indicated on the left side. The arrows on the right side indicate the positions and size (in kD) of cleaved forms of αA, αB, βB2 and γ-crystallins in the NaOH-insoluble proteins from α3 (-/-) lenses.

Figures 8A-8C show the coding nucleotide sequence of mouse α3 connexin gene and the encoded protein. The nucleotide and amino acid sequences are listed in SEQ ID NO 1.

Detailed Description of the Invention

A. Definitions

Knockout: A partial or complete suppression of the expression of at least a portion of a protein encoded by an endogenous DNA sequence in a cell.

Knockout Construct: A nucleic acid sequence that is designed to decrease or suppress expression of a protein encoded by endogenous DNA sequences in a cell. The nucleic acid sequence used as the knockout construct is typically comprised of (1) DNA from some portion of the gene (exon sequence, intron sequence, and/or promoter sequence) to be suppressed and (2) a marker sequence used to detect the presence of the knockout construct in the cell.

Disruption of the Gene or Gene Disruption: Insertion of a nucleic acid sequence into one region of the native DNA sequence (usually one or more exons) and/or the promoter region of a gene so as to decrease or prevent expression of that gene in the cell as compared to the wild-type or naturally occurring sequence of the gene.

Rodent and Rodents: Includes any and all members of the phylogenetic order Rodentia including any and all progeny of all future generations derived therefrom.

Murine: Includes any and all members of the family Muridae, including rats and mice.

Progeny or Offspring: Includes any and all future generations derived and descending from a particular mammal, i.e., a mammal containing a knockout construct inserted into

its genomic DNA. Thus, progeny of any successive generation are included herein such that the progeny, the F1, F2, F3 generations and so on indefinitely are included in this definition.

Analog: Refers to a molecule substantially similar in function to interleukin-1β converting enzyme (ICE).

Homolog: Refers to a molecules that is structurally or functionally equivalent to a molecule of the present invention.

Therapeutically Effective Amount: Refers to an amount effective in treating or ameliorating a cataract condition in a subject characterized by growth including formation, progression and regression.

Prophylactically Effective Amount: Refers to an amount effective in preventing the formation of a cataract.

Isolated: Refers to a state or condition that is not the natural state, i.e., that, if it occurs in nature, it has been changed or removed from its original environment or both.

B. Transgenic Non-Human Mammals Presenting with Cataracts Following Disruption of α3 Connexin Gene

1. Preparation of α3 Connexin Gene-Disrupted Non-Human Mammals

Methods of making disrupted genes and mammals containing such genes are well known to one of ordinary skill in the art. The present invention is based in part on the ability to decrease or completely suppress the level of expression of a particular gene in a mammal by introducing into the genomic DNA of that mammal a new exogenous DNA sequence that serves to interrupt some portion of the endogenous DNA sequence to be suppressed. Another term for this type of suppression is "knockout". Thus, the exogenous

nucleic acid is also referred to as a "knockout construct".

Typically, as described in the present invention, the knockout construct, in this case the plasmid or expression vector referred to as α3 connexin targeting vector or pα3-GZK described below and in the Examples, is first prepared and then inserted into an embryonic stem cell in which the construct subsequently becomes integrated into that cells' genomic DNA, usually by the process of homologous recombination. The embryonic stem cell containing the exogenous DNA is then subjected to selection methods as described in the Examples, for example, by selection for neomycin resistance. Selected resistant cells are then analyzed for the presence of a mutant allele. An embryonic stem cell containing a mutant allele is then injected into a blastocyst that is implanted into the uterus of a pseudopregnant foster mother for integration into a developing embryo.

Offspring that are born to the foster mother are then screened for the presence of the mutant allele, for example, as is described more completely in the Examples. Preferably, the mutant allele is present in the germ line allowing for the generation of homozygous mutant animals from crossing heterozygotic animals containing the desired mutant allele.

Exemplary teachings of the preparation of disrupted genes and mammals containing such genes are provided in US Patent Nos. 5,553,178, 5,557,032 and 5,569,824, the disclosures of which are hereby incorporated by reference. The α3 connexin-deficient non-human mammals of this invention are generated essentially as described in the outlined procedure above and in the Examples. Another term for α3 connexin is Cx46. Thus, the invention provides a mammal, preferably a mouse, and its progeny either containing a nonfunctional α3

connexin protein or lacking functional α3 connexin protein altogether. The resultant mammal is referred to as a α3 connexin-deficient mammal. The α3 connexin deficiency is generated by the insertion of a nucleic acid sequence comprising at least a portion of the α3 connexin gene into the genome of the mammal. The inserted α3 connexin nucleic acid sequence present in the genome is referred to as a disrupted α3 connexin gene. Exemplary disrupted α3 connexin gene nucleic acid sequences from non-human mammals are discussed in Example 1.

In preferred embodiments, a disrupted α3 connexin gene lacks at least one transmembrane-encoding region and more preferably two regions and most preferably four regions. In a further embodiment, an α3 connexin gene lacks one or more nucleotides in the protein coding region, the presence of which results in a nonfunctional α3 connexin protein. In another embodiment, an α3 connexin gene contains one or more nucleotide substitutions, the presence of which also results in a nonfunctional α3 connexin protein.

In another aspect, the invention provides a nucleic acid sequence corresponding to the above-described disrupted α3 connexin gene operably linked to a plasmid vector also referred to as a targeting vector. A preferred plasmid comprises a nucleic acid sequence that is an α3 connexin gene lacking four transmembrane domains. An α3 connexin gene-containing plasmid further comprises at least a selectable marker, preferably the gene encoding neomycin resistance. In other embodiments, an α3 connexin gene-containing plasmid also comprises a marker gene for use in detecting the presence of an α3 mutant allele in a mammal of this invention. A preferred marker gene encodes lacZ. A preferred disrupted α3 connexin gene-containing plasmid is the plasmid, designated

pα3-GZK, on deposit with American Type Culture Collection having ATCC Accession Number 97848, the deposit of which is further described below and in Example 4. In further aspects, the invention provides an embryonic stem cell, preferably a JS-1 or an ES cell, containing a disrupted α3 connexin gene.

Exemplary expression systems comprising suitable vectors and host cells for use in the present invention are described in Example 1 and further supported by ICE-related US Patents 5,552,536, 5,578,705 and 5,654,146, the disclosures of which are hereby incorporated by reference. Methods for introducing a disrupted α3 connexin nucleic acid into a recipient host cell is described in Example 1 and further supported by all of the above-identified US Patents, including those describing preparation of transgenic knock-outs.

The system and methods can be practiced with any non-human α3 connexin genes, and therefore the invention need not be limited to the example of the mouse models as described above. Furthermore, as previously discussed, a disrupted α3 connexin gene is not limited to only one type of disruption in which the four transmembrane-encoding regions have been deleted. Thus, any α3 gene disruption that results in the generation of a nonfunctional but present α3 connexin protein or that blocks the transcription or translation for such protein is contemplated for use in generating an α3 connexin-deficient mammal.

2. Characterization of α3 Connexin Gene-Disrupted Non-Human Mammals

The introduction of a disrupted α3 connexin nucleic acid of the present invention described above and in the Examples into a recipient host results in a transgenic non-human mammal having a disrupted α3 connexin gene. As a result

of the α3 connexin gene disruption, the non-human mammal exhibits unique characteristics summarized herein and further discussed in Example 1.

Although the absence of α3 connexin had no obvious influence on the early stages of lens formation and the differentiation of lens fibers, the non-human mammals of this invention homozygous for the disrupted α3 connexin gene developed nuclear cataracts at the age of 2 to 3 weeks. Gap junctions containing α3 connexin were found in the homozygous non-human mammals, but they could not compensate for the functional loss of α3 connexin in the lens nucleus. The nuclear cataracts resulted from light scattering of high molecular weight aggregates formed by the lens proteins linked together via disulfide bonds. A significant amount of degraded crystallins, in particular a unique fragmentation of γ-crystallin, was present in the α3 connexin (-/-) lenses indicating that proteolysis in the lens plays a critical role in the cataractogenesis of the α_3 (-/-) mice.

Thus, in the present invention, the α3 connexin gene is shown to be essential for providing a cell-cell signaling pathway or structural component for maintaining the organization of lens membrane and cytoplasmic proteins that is required for normal lens transparency. Further, the invention provides the discovery that a loss of α3 connexin initiates a process in the lens that may involve an apoptosis pathway.

C. Therapeutic Compositions and Methods to Prevent or Treat Cataracts with Inhibitors of Interleukin-1β Converting Enzyme Activity

The discovery of the γ-crystallin degradation in the lenses of the α3 connexin gene-disrupted non-human mammals of the present invention provides for therapeutic compositions

and methods of use thereof for affecting growth of cataracts in a subject mediated by loss of α3 protein, nonfunction of α3 protein or a degradation of critical crystallin lens proteins. Thus, as discussed in Example 2, a prophylactically or therapeutically effective cataract growth affecting amount of an interleukin-1β converting enzyme (ICE) inhibitor administered to a subject patient. A cataract growth affecting amount of an inhibitor of this invention is any amount that is effective at inhibiting the formation of cataracts in a subject predisposed to cataract formation, such as in the α3 connexin-deficient mammal described herein. Thus, the inhibitor acts prophylactically at preventing cataract formation. In other aspects, a cataract growth affecting amount is any amount that is effective at altering the progression of cataract formation including delaying the complete formation of a cataract, inhibiting the complete formation of a cataract, and reversing a progressing or a completely formed cataract.

An inhibitor of the present invention is one that functions to inhibit the degradation of the lens crystallin proteins, preferably γ-crystallin lens protein. Accordingly, inhibitory compounds of this invention are capable of targeting and inhibiting degradation of lens proteins, the degradation of which plays a critical role in cataract development. Such inhibitors include but are not limited to inhibitors of interleukin-1β converting enzyme (ICE) and analogs thereof. Known ICE inhibitors are well known to those of skill in the art and include but are not limited to Bcl2, Bcl-XL, CrmA (cowpox virus protein), ZVAD-fluromethylketone (a permeant ICE inhibitor), lactacystin, dichloroisocourmarin, peptides Ac-YVAD-CMK and CPP32/Yama, and DEVD-CHO, and those described in US Patents 5,656,627, 5,552,536, 5,672,500 and

5,654,146, the disclosures of which are hereby incorporated by reference.

Exemplary teachings of therapeutic compositions and methods of administration applicable to the present invention are also provided in ICE-related US Patents 5,578,705 and 5,656,627, the disclosures of which are hereby incorporated by reference.

For treament, the candidate inhibitor is administered orally, intravenously, topically, or the like routes. For topical administration to the eye, the compound is preferably providied in association with an opthalmalogically acceptable carrier. Preferred opthamalogical modalities are provided in US Patents 5,401,880, 5,422,376, 5,519,054, 5,578,578 and 5,628,801, the disclosures of which are hereby incorporated by reference.

The effect of the inhibitor is evaluated most easily by direct examination of the lens optometrically.

Thus, the compounds of this invention may be employed in a conventional manner for the treatment of cataractogenesis mediated by the disruption of α3 connexin gene, loss or nonfunction of α3 connexin protein and degradation of lens proteins. Such methods of treatment, their dosage levels and requirements may be selected by those of ordinary skill in the art from available methods and techniques. For example, a compound of this invention may be combined with an acceptable pharmaceutical carrier or an opthalmalogically acceptable carrier for a time period and in an amount effective to affect cataract growth in a subject. Alternatively, the inhibitors of this invention may be used in compositions and methods for treating or protecting a subject against cataractogenesis over extended periods of time. An inhibitor may be employed in such compositions either alone or together with other

inhibitors of this invention in a manner consistent with the conventional utilization of ICE inhibitors in pharmaceutical compositions. For example, an inhibitor of this invention may be combined with pharmaceutically acceptable carriers conventionally employed in vaccines and administered in prophylactically effective amounts to protect individuals over an extended period time against acquiring cataracts.

The inhibitors of this invention may also be co-administered with other ICE inhibitors to increase the effect of therapy or prophylaxis against cataractogenesis. When the inhibitors of this invention are administered in combination therapies with other agents, they may be administered sequentially or concurrently to the subject. Alternatively, pharmaceutical or prophylactic compositions according to this invention may be comprised of a combination of an ICE inhibitor of this invention and another therapeutic or prophylactic agent.

Pharmaceutical and opthalmalogical compositions of this invention comprise any of the compounds of the present invention, and pharmaceutically/opthalmalogically acceptable salts thereof, with any pharmaceutically acceptable carrier, adjuvant or vehicle. As used herein, the terms "pharmaceutically acceptable", "opthalmalogically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as burning, irritation, shock, nausea, dizziness, gastric upset and the like. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not

be limited based on formulation.

Pharmaceutically/opthalmalogically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride-mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts,colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxy-methylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally,buccally, vaginally or via an implanted reservoir. The preferred route is topically directly to the eye for either pharmaceutical or opthalmalogical formulations. Such formutions are well known in the art using suitable dispersing or wetting agents and suspending agents. The formulations are preferably sterile solutions or suspensions in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or

diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions.

A therapeutically effective amount of large molecule inhibitors of this invention is typically an amount such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.01 microgram (μg) per milliliter (ml) to about 100 μg/ml, preferably from about 1 μg/ml to about 5 μg/ml, and usually about 5 μg/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, preferably from about 0.2 mg/kg to about 200 mg/kg, most preferably from about 0.5 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days.

A therapeutically effective amount of a small molecule inhibitors of this invention is typically an amount of polypeptide such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram (μg) per milliliter (ml) to about 200 μg/ml, preferably from about 1 μg/ml to about 150 μg/ml. Based on a polypeptide having a mass of about 500 grams per mole, the preferred plasma concentration in molarity is from about 2 micromolar (μM) to about 5 millimolar (mM) and preferably about 100 μM to 1 mM polypeptide antagonist. Stated differently, the dosage per body weight can vary from about 0.1 mg/kg to about 300 mg/kg, and preferably from about 0.2 mg/kg to about 200 mg/kg, in one or more dose administrations daily, for one or several days.

D. Screening Methods to Identify Inhibitors of Interleukin-1β Converting Enzyme Activity Useful in Preventing or Treating Cataracts

The discoveries of the present invention also provide a variety of screening methods both *in vivo* and *in vitro*. In general, the screening methods are useful in identifying inhibitors of ICE or ICE-like activity occurring in the lens that may be responsible for the generation of cataract growth.

Thus, in one embodiment,a screening method to identify drugs that block, diminish or reverse the development or severity of cataracts relies on using the α3 connexin-deficient mouse model of this invention. This method involves the administration of a candidate drug over a range of doses to the mouse by various routes of delivery, preferably by eye drops or intravencusly. Exemplary teachings are in Example 2.

In another screening method, the effect of a compound on a lens is also assessed through an *in vitro* system in which the lens of the mouse described above is cultured according to methods well known in the art for explant organ culture. The compound is directly applied to the medium in which the lens is cultured. Effects on opacification are assessed visually, morphologically or biochemically as previously described.

The present invention also describes methods to identify an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog. Inhibitors are identified with a variety of approaches. Two exemplary methods utilize the non-human mammal of the present invention and the discovery of the degradation pattern of the γ-crystallin lens protein as a result of the α3 connexin gene disruption in the non-human mammal.

In one method, a γ-crystallin lens protein is contacted with a solution of ICE or functional ICE analog to form a

mixture in the presence of a candidate inhibitor of ICE or functional ICE analog under conditions described in Example 1 for assessing cleavage patterns of γ-crystallin. The effect of the inhibitor is then assessed by comparing the cleavage patterns of untreated and treated samples. An effective inhibitor is identified with the presence of an altered cleavage pattern compared to that of undegraded γ-crystallin.

For these assays, the lens protein is in either purified or unpurified form. The ICE or functional ICE analog is either commericially obtained or is providedin a homogenate of lens isolated from the non-human mammal as described in Example 1.

Another screening method to identify an inhibitor of ICE or a functional ICE analog is based on the use of an ICE or functional ICE homolog substrate well known to those of skill in the art of apoptosis. Exemplary ICE substrates include γ-crystallin and precursor interleukin-1β (pIL-1β) and are described in US Patents 5,607,831 and 5,656,627, the disclosures of which are hereby incorporated by reference. The substrate is mixed with a homogenate of lens isolated from the non-human mammal prepared in Example 1. The homogenate contains ICE or functional ICE analog as described in Example 1 that results in the novel cleaveage pattern of γ-crystallin. The above mixture is prepared in the presence of a candidate inhibitor of ICE or functional ICE analog under conditions where ICE or functional ICE analog is known to cleave the substrate generating detectable cleavage products. The cleavage pattern obtained with the test compound is compared to that with a control thereby identifying the effectiveness of the test compound as an inhibitor of the novel protease in the lens. Exemplary teachings of screening methods applicable to the present invention are provided in US Patents 5,656,627.

In a further aspect relating to the identification of an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog, the present invention contemplates a kit containing packaging means that include a first container containing a γ-crystallin lens protein in an amount sufficient for at least one assay, and further including a packaging material comprising a label indicating that the crystallin lens protein can be used to identify the inhibitor by detection of an altered cleavage pattern. Exemplary teachings of the cleavage patterns are provided in the Examples. The enzymatic component of the kit is further provided in the form of a second container having ICE or functional ICE analog that is either commerically available or is present in a homogenate of lens isolated from a non-human mammal of this invention. Preparation of such homogenates are detailed in the Examples.

The invention further contemplates a kit for identifying an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog in which a substrate, preferably a γ-crystallin lens protein, of ICE or functional homolog and a homogenate of lens isolated from a non-human mammal of this invention having disrupted alleles of α3 connexin gene are provided in separate containers. The kit further contains a packaging material comprising a label indicating that the crystallin lens protein can be used to identify the inhibitor by detection of an altered cleavage pattern.

Examples

The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed,

which would be within the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

1. Preparation and Characterization of a Non-Human Mammal Having a Disrupted α3 Connexin Gene

A. Experimental Procedures

1) Construction of α3 Targeting Vector and Generation of α3 Homozygous (-/-) Mice

In order to generate α3 connexin knockout mice, an α3 targeting vector containing a disrupted α3 connexin gene was prepared. Two α3 connexin genomic clones encompassing a total of 25 kilobases (kb) of DNA sequence for the α3 connexin gene locus were isolated from a 129 FIX library (Stratagene, La Jolla, CA) according to manufacturer's instructions. The full-length α3 connexin protein, like all other members of this family, is encoded entirely within a single exon. The physical map of the wild type α3 gene locus is shown in Figure 1A.

The nucleotide sequences of mouse, rat and bovine α3 (also referred to as Cx46) connexin genes are available with the respective GenBank Accession Numbers U44955, X57970, and U08213. The nucleotide sequence of the mouse α3 connexin gene is 2770 nucleotides with the protein coding sequence corresponding to nucleotide positions 388-1641. The nucleotide coding sequence and translated amino acid sequence are shown in Figure 8 and listed in SEQ ID NO 1. Thus, the 5' untranslated sequence in the genomic sequence has been deleted for Figure 8. In this format, Figure 8 provides easy reference to the regions of the four transmembrane domains (TMDs) that occur approximately at amino acid residues 24-41, 77-94, 150-168, and 208-227. The nucleotide sequence regions

corresponding to the four TMDs in Figure 8 are the following respective nucleotide regions: 70-123, 229-282, 448-504, and 622-681. Homologous regions from other non-human mammalian species are readily determined as well known to one of ordinary skill in the art.

An isolated disrupted α3 connexin gene nucleic acid sequence of the present invention can be prepared in many ways well known to one of ordinary skill in the art of gene expression and manipulation of DNA. Any methodology is acceptable such that the result is an absence of expression of α3 connexin protein or an absence of expression of functional α3 connexin gene. In other words, either no protein is translated or a nonfunctional α3 connexin protein is translated. Thus, for blocking the production of α3 connexin altogether, the entire α3 connexin gene can be deleted, while at the other extreme, a means to inhibit the transcription and translation of the protein are equally effective.

For preparing expressed but nonfunctional α3 connexin protein, various regions of the α3 connexin protein can be deleted at the nucleotide level thereby interfering with the ability of the protein to perform its intended function as a gap juntion forming protein. In this aspect, the transmembrane domains of α3 connexin protein are thought to be critical for the creation of functional gap junctions. As discussed above, four TMDs exist in α3 connexin protein. At a minimum, a disrupted α3 connexin gene encoding a nonfunctional α3 connexin protein is generated by deletion of at least one TMD. Preferably, the disruption encompasses at least two TMDs and more preferably, four TMDs as described below. Approaches to effect a disruption of a TMD is to delete the corresponding nucleotide sequence or to insert a termination codon. An alternative approach is to delete a region and replace with an

exogenous sequence. This approach was used as described below in which the reporter *lacZ-nls* sequence was inserted at approximately nucleotide positions shown in Figure 8 from 73 through 681 effecting a deletion of four TMDs while terminating the translation of the remainder of the α3 connexin gene. Note that the nucleotide position numbers are not identical to those in the GenBank sequence as the 5' untranslated sequence has been deleted for convenience in referring to regions of the α3 connexin gene and encoded protein. Primers as described below were designed from the complete GenBank sequence.

To prepare a disrupted α3 connexin gene as shown in Figure 1A, two fragments from the genomic clones were obtained by restriction digest. As a result, an internal deletion corresponding to the region encoding four transmembrane domains of the α3 connexin protein was generated in exon 2 of the α3 connexin gene. The nucleotide region between the Hgi AI site and the downstream Apa I site in the α3 connexin gene coding region was eliminated as a result of the cloning procedures outlined herein.

A 4 kb Eco RI-Hgi AI α3 fragment for α3 connexin containing 5' untranslated sequence along with sequence encoding the first 17 amino acid residues was ligated in-frame with the nuclear location sequence (nls) and a lacZ reporter gene (Bonnerot et al., Proc. Natl. Acad. Sci., USA, 84:6795-6799 (1987) through a linker sequence. This cloning procedure to obtain the resultant vector, referred to as pα3-GlacZn, was performed through intermediate vectors pα3-GL and pLacF, the latter of which provided the lacZ sequence. The vector pα3-GlacZn was then digested with Not I to release a fragment containing the 5' end of α3 fragment operably linked to the lacZ-encoding sequence. This α3-lacZ fragment was inserted

into a similarly digested expression vector referred to as pPNT (Tybulewicz et al., Cell, 65:1153-1163 (1991).

To complete the disrupted α3 connexin vector, a 3.7 kb Apa I-Hind III α3 fragment containing the rest of exon 2 from the genomic clones was isolated and then inserted into the BamHI site of pPNT containing the 5' end of the gene by blunt end ligation to generate the α3 connexin targeting vector. As a result of the ligation sites into the pPNT vector, the α3 connexin gene regions were separated by a pGK-neomycin gene. The resultant vector, designated pα3-GZK, is shown in Figure 1A with the respective indicated inserts described above. The pα3-GZK vector was deposited with the American Type Culture Collection as described in Example 4.

Alternative expression vectors are used to prepare disrupted α3 connexin vectors for use in generating non-human mammals having a mutant allele for a disrupted α3 connexin gene. Alternative constructs for generating an α3 connexin deficiency are generated by the insertion into a non-human mammal of a nucleic acid sequence comprising at least a portion of the α3 connexin gene into the genome of the mammal. Other disrupted α3 connexin gene constructions lack at least one transmembrane-encoding region, one or more nucleotides in the protein coding region of exon 2, or lack one or more nucleotides in the protein coding region. These constructs are readily obtained by using procedures described herein as well as those known to one of ordinary skill in the art.

The targeting plasmids prepared above were then transfected into J1 embryonic stem cells (ES) by electroporation. Neomycin-resistant clones were verified by Southern blot. Two of these α3 knockout clones were injected into C57BL/6J blastocysts to produce chimeras as described (Li

et al., Cell, 69:915-926 (1992)). Male chimeras were used as founders to breed F1 hybrids with C57BL/6J female mice. F2 mice were generated from the intercross between α3 heterozygous (+/-) F1 mice.

For Southern blot analysis, the DNA was isolated from ES cells or from mouse tails, then digested with Bam HI. A 900 base pair (bp) Eco RI fragment (probe A in Figure 1A), next to the 5' homologous region in the α3 gene locus was used as the probe to examine the size of the wild type or α3 gene disrupted alleles. The procedure for Southern blot analysis followed the protocol provided by the manufacturer (GeneScreenplus membranes, NEN Research Products). Three primers were used for PCR to examine the wild type or α3 mutant alleles: the oligo deoxyribonucleotide 5'-CCCAGGCTCTACCTCAGGTT3' (SEQ ID NO 2) was used as a 5' primer for detecting both the wild type allele and the α3 knockout allele; the oligo deoxyribonucleotide (5'-CTTTGCCGATGACTGTAGAG-3' (SEQ ID NO 3) was used as the 3' primer for detecting the wild type gene allele; and the oligo deoxyribonucleotide (5'-CAGGGTTTTCCCAGTCACGAC-3' (SEQ ID NO 4) from the lacZ gene sequence was used as the 3' primer for detecting the α3 gene disrupted allele. The expected PCR products from the wild type allele or the α3 disrupted allele, respectively, were a 350 bp or a 500 bp band.

For Northern blot analysis, total RNAs were isolated from 10 to 14 lenses per group from α3 (+/+), (+/-), or (-/-) mice at the age of one month, by the CsCl method (Sambrook et al., Molecular Cloning, A Laboratory Manual, S. Edition, ed: CSH, (1989)). The Northern blot analysis followed the protocol provided by the manufacturer (GeneScreenplus membranes, NEN Research Products). An α3 cDNA probe, a 0.8 kb Hgi A1 to Apa I fragment encoding the four transmembrane domains of the α3

connexin protein, was used for hybridization.

2) LacZ Staining, Histological and Immunocytochemical Analysis

The lacZ staining method was carried out as described (Bonnerot and Nicolas, In Methods of Enzymology, P.M. Wassarman and M.L. DePamphilis, eds., pp. 451-469 (1993)). Lenses from embryos or newborn mice were prepared by using a standard histological method for paraffin-section and hematoxylin and eosin staining. Lenses from adult mice were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 5 days, and than embedded in LR white resin. Sections were stained with methylene blue. For immunocytochemical staining, mouse eyes were fixed with fresh 4% formaldehyde in phosphate-buffered saline (PBS) for 30 minutes, then washed with PBS twice, and soaked overnight in 30% sucrose in PBS. Afterwards, the samples were processed by using a standard frozen-section method and analyzed with different antibodies as described (Risek et al., Develop. Biol., 164:183-196 (1994)). The distribution of antigen was analyzed by laser scanning confocal microscopy (Bio-Rad, C600). For freeze-fracture electron microscopy, the lenses were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 5 days, then processed and analyzed as described previously (Risek et al., id., (1994)).

3) Gel Filtration Analysis

Fresh lenses were dissected from the mice, and homogenized in 0.1 M NaCl, 50 mM Na_2HPO_4 (pH 7.0) at 40 mg lens wet weight/ml solution. The soluble fraction was collected after 15 minutes of centrifugation at 15000 rpm, and filtered through a microfilterfuge tube (0.45 μm NYLON-66, Rainin).

Gel filtration analysis was then carried out on a Pharmacia-LCC 500 instrument with a Superose 6 HR 10/30 column. The elution buffer was 50 mM Na_2HPO_4, 0.1 M NaCl (pH 7.0).

4) Biochemical and Western Blot Analysis

To prepare the NaOH-insoluble protein samples, lenses were dissected from both eyes of an animal, and homogenized in 0.5 ml of 20 mM NaOH and 1 mM Na_2CO_3. The insoluble pellet was collected after centrifugation at 15000 rpm for 15 minutes, washed once with the same solution, and then washed once with 1 mM Na_2CO_3. The insoluble pellets were then dissolved in an equal volume of sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol and 0.001% bromophenol blue) with or without 10 mM DTT. When DTT was omitted to avoid reducing proteins to subunits, 10 mM iodoacetamide was included to prevent disufide interchange.

To prepare the lens samples for Western Blot analysis, each lens was separated into two parts: a lens nucleus (around 60% of total lens wet weight) and a lens cortex (the remaining part). The lens cortex was dissolved in sample buffer containing 10 mM DTT; this is referred to as the total lens cortex proteins. The lens nucleus was extracted three times with 0.1 M NaCl, 50 mM Na_2HPO_4; the supernatant represented the total water-soluble proteins in the nucleus. The insoluble pellet was further extracted three times with 20 mM NaOH, 1 mM Na_2CO_3; this supernatant represented the NaOH-soluble protein. Finally, the insoluble pellet was dissolved in sample buffer with 10 mM DTT, and this is denoted as the NaOH-insoluble proteins. All samples for Western blot analysis were dissolved in loading buffer with 10 mM DTT.

The 9 and 11 kilodalton (kDa) degraded γ-crystallins were separated from the NaOH-insoluble proteins of α3 (-/-) lenses

by a 4-20% polyacrylamide pre-cast gradient gel (Novex) and transferred to a PVDF (BioRad) membrane. N-terminal sequence analysis was performed by Edman degradation on a Perkin-Elmer/Applied Biosystems Model 494A sequencer.

B. Results

1) Production of an α3 Knockout Mouse

As previously discussed, the physical maps of the wild type α3 gene locus, the α3 connexin gene targeting vector (pα3-GZK), and the disrupted α3 gene locus are schematically shown in Figure 1A. This targeting plasmid, pα3GZK, was then transfected into J1 embryonic stem cells (ES cells). Eighteen out of 240 neomycin-resistant clones were confirmed to have undergone homologous recombination of their DNA as determined by Southern blot analysis. A 12 kb band that corresponds to the α3 wild type allele and a 5.5 kb band to the mutant allele were detected (Figure 1B). Two independent ES cell clones whose DNA showed the 5.5 kb band were used to generate 11 male chimeras. Genomic DNA from both F1 and F2 mice was analyzed by Southern blot (Figure 1C) or PCR (Figure 1D). The α3 connexin genotype from 196 F1 and 312 F2 mice followed Mendelian rules (data not shown). Both α3 (+/-) and α3 (-/-) mice were as viable and fertile as wild type. The connexin α_3 transcripts were detectable in α3 (+/+) and α3 (+/-) mice, but not α3 (-/-) (Figure 1E). The level of α3 connexin transcripts in α3 (+/-) mice was 40 to 70% of that of α3 (+/+) mice as measured by densitometry, consistent with the presence of only one connexin gene allele in the heterozygotes.

2) Expression Pattern of the *lacZ-nls* Reporter Gene During Lens Development

Analysis of the expression pattern of the *lacZ-nls* gene, which contains a nuclear localization signal, from both α3 (-/-) and α3 (+/-) mice showed that *lacZ-nls* activity was first detected in the primary lens fiber cells of the embryos at stage 11 dpc, and in the secondary fibers throughout the life of the mice. Whole-mount staining for β-galactosidase from α3 (+/+), α3 (+/-) and α3 (-/-) embryos at stage 14 dpc is shown (Figure 2A: left embryo, α3 (+/+); middle embryo, α3 (-/-); right embryo, α3 (+/-)). A typical lacZ staining histological section along the lens equatorial plane of an α3 (-/-) embryo at stage 14 dpc is also shown (Figure 2B). The α3 (+/-) embryos contained an identical lacZ staining pattern in the lens as in the α3 (-/-) embryos, but the level of staining was lower than in the α3 (-/-). In sections from the lens of α3 (-/-) mice at the age of two weeks, β-galactosidase activity was detected in the nuclei of lens fiber cells, and also at a lower level in the nuclei of lens epithelial cells (Figure 2C, arrows). The newly formed secondary fiber cells in the bow region showed the highest intensity of staining. In the deep cortical and nuclear region the lacZ staining decreased and eventually disappeared probably due to the elimination of the cell nuclei along with other intracellular organelles during maturation of the lens fiber cells. A low level of β-galactosidase activity was also detected in the tip of the limbs during embryonic stages 11.5 to 14.5 dpc and in the dorsal root ganglion cells after stage 13.5 dpc (data not shown).

3) Histological Analysis of Lens Development

Lens development of the α3 (+/+), α3 (+/-) and

α3 (-/-) mice was examined by standard histological methods. From embryonic stage 11 dpc to postnatal day 3, no significant morphological changes were found in α3 (-/-) mice. Typical examples are shown in the histological sections from an α3 (-/-) (Figure 2D) and an α3 (+/+) (Figure 2E) embryo at stage 18.5 dpc. However, beginning at 2-3 weeks of age, and increasing with age, an abnormal punctate light scattering was observed with a dissection microscope in the nuclear region of the lenses from α3 (-/-) mice. This nuclear cataract was observed in the lenses of all the α3 (-/-) mice examined; i.e., it had a 100% penetrance. The degree of the nuclear opacity varied somewhat among individual mice, but the nuclear cataracts in the two lenses from the same mouse were identical. No nuclear cataracts were observed in α3 (+/+) and α3 (+/-) mice up to the age of 8 months.

Figure 3A contains an α3 (+/+) lens and an α3 (-/-) lens from mice at the age of 2 months. The light scattering opacity in the lens is commonly referred to as a cataract. The cataract in α3 (-/-) lenses resulted from the light scattering of many aggregates in the lens nucleus; the lens cortex remained transparent. Histological analysis of the cortical fibers in the sections from the α3 (-/-) cataract lens showed normal morphology with no swelling (Figure 3B), but regions with aggregated material (arrow) were observed in the nuclear region (Figure 4A).

4) Characterization of α3 and α8 Connexins in the Lens

The localization of α3 and α8 connexin proteins in the mouse lens membrane was characterized by using an anti-α3 connexin peptide antibodies and anti-α8 connexin monoclonal antibodies. In the lens cortex of α3 (+/+) mice, α3 (Figure

4B, left green panel) and α8 (Figure 4B, right red panel) connexins were colocalized to the same plaques (Figure 4B, middle orange panel), and they were located on the broad side of the membranes of the hexagonal fibers. In the cortex of α3 (-/-) mice, the plaque-like staining signals for α8 connexin on the broad side of the membranes of hexagonal lens fibers were still present (Figure 4C, right panel), but the α3 connexin protein was undetectable (Figure 4C, left panel). The plaque-like staining signals of α3 connexin appeared to fade away more steeply from the superficial cortex to the deep cortex in the α3 (-/-) mice than in the α3 (+/+) mice.

5) Electron Microscopic Analysis of the Lens Membranes

Gap junction plaques were observed by freeze-fracture electron microscopy in both normal and α3 (-/-) mice, and they were compared in membranes of superficial cortical fibers of lenses from 3 week old mice (Figure 5A). In both animals, the gap junctions exhibited similar pleomorphic variations in plaque sizes and distributions. However, the gap junction particles in the α3 (-/-) lens cortex appeared to be packed less tightly (Figure 5B) than those in the α3 (+/+) lens cortex (Figure 5A), based on a qualitative analysis. In the lens nuclear region, however, the normal polygonal arrays of furrowed membrane structures seen in α3 (+/+) mice (Figure 5C) were completely disrupted in α3 (-/-) lenses (Figure 5D). Strikingly, large areas of continuous square arrays of 5 to 6 nm particles were often detected in the deep cortex and nucleus of α3 (-/-) lenses (Figure 5F). In contrast, particles in square arrays in the membranes of the α3 (+/+) lens nucleus were found as small islands mixed with 9 to 11 nm particles (Figure 5E).

6) Lens Growth Rate and Weight

The wet weight of lenses from α3 (+/+) and α3 (-/-) mice from the age of 3 weeks to 1 year is presented in Figure 6A. There was no significant change in the lens growth rate beween α3 (+/+) and (-/-) mice after birth. Although the average lens weight from the α3 (-/-) mice was 10% less than their wild-type littermates after one year, this difference is within the standard deviation.

7) Characterization of Crystallin Proteins

The total water-soluble protein in lenses from α3 (-/-), α3 (+/-) and α3 (+/+) littermates at different ages was determined by gel filtration. Since more than 90% of the water-soluble protein in the normal lens consists of α, β and γ-crystallins, the focus was on these proteins. At the age of two weeks, α3 (-/-) mice had elution profiles that were identical to those of α3 (+/+) littermates (data not shown). However, by two months of age, α3 (-/-) mice had 15% less water-soluble protein than their α3 (+/-) and α3 (+/+) littermates, although the lenses were identical in wet weight. The elution profiles indicate that the loss of water-soluble γ-crystallin proteins in the lenses of α3 (-/-) mice accounts for most of the deficit (Figure 6B). By 4 months of age, there was a reduction of 30% in the quantity of water soluble proteins in the lenses of α3 (-/-) mice compared to the α3 (+/+) mice. The elution profiles from α3 (-/-) lenses indicated that this was due to the loss of not only γ-crystallins, but also α- and β-crystallins (Figure 6C). Lenses selected for the same total wet weight from 4 month old α3 (-/-) and α3 (+/+) littermates were separated into cortex and nucleus before extraction and analysis. The elution profiles indicated no significant changes of water-soluble

crystallins in the cortex of the α3 (-/-) mice compared to α3 (+/+) mice (Figure 6D); a significant loss of the water soluble α-, β- and γ-crystallins occurred only in the nucleus (Figure 6E).

In general, the proteins that remain insoluble after extraction of normal lenses with 20 mM NaOH in 1 mM Na_2CO_3 solution are membrane proteins. These NaOH-insoluble proteins from α3 (-/-), α3 (+/-) and α3 (+/+) lenses at the age of 2 months were analyzed by Coomassie blue stained SDS-PAGE (Figure 7A). The major NaOH-insoluble protein band from α3 (+/+) and α3 (+/-) lenses was located at the position corresponding to the major lens membrane protein, MP26 (Figure 7A, lanes 1-4). There was a 5- to 10-fold increase in the total amount of NaOH-insoluble protein in α3 (-/-) lenses compared to α3 (+/-) and α3 (+/+) lenses. The NaOH-insoluble proteins of α3 (-/-) lenses were analyzed by SDS-PAGE in the presence and absence of DTT. After treatment with DTT, most of these insoluble proteins were located between 17 and 27 kDa (Figure 7A, lane 5); much less protein was detected in the same range when the insoluble proteins were not treated with DTT (Figure 7A, lane 6). This is likely due to the fact that the NaOH-insoluble proteins from α3 (-/-) lenses consisted of high molecular weight aggregates of lens proteins linked together via disufide-bonds *in vivo*; without DTT treatment, these aggregates were not able to enter a 5% polyacrylamide gel.

Lenses were physically dissected to obtain nuclear regions (around 60% of total lens weight) and cortical regions (the remaining 40%). The lens nuclear region was then subdivided into three fractions: water-soluble; NaOH-soluble; and NaOH-insoluble fractions. All fractions were analyzed on Western blots using antibodies against αA-,

αB-, β2- and γ-crystallin. Results of samples from 2 month old α3 (+/+) and α3 (-/-) mice are shown in Figure 7B. Only one significant difference was observed in the lens cortex: an increase in the cleaved form of αB-crystallin in α3 (-/-) mice (Figure 7B, lane 5) compared to α3 (+/+) mice (Figure 7B, lane 1). In the lens nucleus, water-soluble αA-, αB-, βB2- and γ-crystallins from α3 (-/-) mice (Figure 7B, lane 6) were similar to that of α3 (+/+) mice (Figure 7B, lane 2). The NaOH-soluble proteins from both α3 (+/+) and α3 (-/-) lens nuclei contained the cleaved forms of αA- and αB-crystallins (lane 3, and 7 in Figure 7B). However, no crystallins were detected in the NaOH-insoluble fraction prepared from the nuclear region of α3 (+/+) lenses (Figure 7C, lane 4). In contrast, the pellet fraction from α3 (-/-) lenses contained a significant amount of the cleaved forms of αA-crystallin, αB-crystallin, βB2-crystallin, the intact form of γ-crystallin and an 11 kDa cleaved form of γ-crystallin (Figure 7B, lane 8 indicated by arrows). Thus, most of the NaOH-insoluble proteins of α3 (-/-) lenses in lane 5 of Figure 7A were likely to be insoluble crystallins since they migrated in the range of 17 to 27 kDa.

Most of the cleaved forms of αA- and αB-crystallin in the α3 (-/-) lens were similar to the sizes of the cleaved products of αA- and αB-crystallins in the α3 (+/+) lens nucleus. However, a unique 11 kDa cleaved γ-crystallin band was detected only in the nuclear region of α3 (-/-) lenses, where it comprised 5-10% of the total insoluble γ-crystallin in the 1 month old cataract lens. The 11 kDa γ-crystallin fragment was first detected in the lenses of 3 week old α3 (-/-) mice, and its detection was correlated with the appearance of the nuclear cataract. The degree of nuclear opacity was directly related to the detectable quantity of

this 11 kDa γ-crystallin fragment (data not shown).

Two γ-crystallin fragments (indicated by arrows in Figure 7A, lane 5), the 11 kDa fragment and a second band of 9 kDa, were purified by 4-20% PAGE from the NaOH-insoluble proteins of α3 (-/-) lenses. The N-terminal sequence of the 11 kDa fragment was identified by Edman degradation to be SI(V)RSFR which is similar to residues 74 to 79 in γ-crystallin. The N-terminal sequence of the 9 kDa fragment was GKITFY, identical to the N-terminal sequence of intact γ-crystallins. Therefore, it is reasonable to predict that a cleavage site for γ-crystallins is located between Asp73-Ser74. A single proteolytic cleavage at this position would generate two fragments with the same precise sizes and the identical N-terminal sequences observed in the α3 (-/-) lenses.

C. Discussion

In the present invention, the α3 (Cx46) connexin gene was disrupted in mice by homologous recombination. Mice homozygous (-/-) for the disrupted α3 gene developed a nuclear cataract that resembled the age-dependent senile cataracts in humans. This phenotype, based on the function of gap junctions, results from alterations in cell-cell signaling pathways. In addition, it was observed that the α3 heterozygous (+/-) and homozygous (-/-) knockout mice were viable and fertile, and the early stages of lens formation and differentiation appeared to be normal.

Morphological and biochemical analysis of lenses from the homozygote mice indicated significant changes when compared to lenses from both heterozygote or normal mice. The phenotype of these cataractous lenses were documented by histological, immunocytochemical and electron microscopic analysis. The biochemical analysis of these lenses indicate that the nuclear

cataract resulted from aggregation of lens proteins that are linked by disulfide bonds. Further, this aggregation of lens protein was initiated and facilitated by degradation of crystallins and, in particular γ-crystallin. The single specific cleavage site determined for γ-crystallin indicates that the protease involved is a potential member of the caspase family which have been implicated in apoptosis (Patel et al., FASEB J., 10:587-597 (1996). Thus, in the present invention, the loss of α3 connexin initiates a pathway in the lens that is normally involved in lens fibre maturation and which has common features to those found in apoptosis.

Although the precise relationship between the lack of α3 connexin and cataractogenesis has not been defined, the results of the present invention indicates that cataractogenesis results from one of two potential functions for α3 connexin. First, in the absence of α3 connexin, the small molecules that are normally transmitted via the α3 connexin pathways and that have a regulatory influence on the activity of molecules, such as proteases, are not able to pass from cell to cell. Consequently, the protease activity is not regulated normally so that fragmentation of molecules, such as the γ-crystallin, occurs abnormally to promote the cataract phenotype. Second, the α3 connexin may make significant contribution as an integral membrane protein for lens membrane/cytoskeletal organization. In the absence of α3 connexin in this superstructural organization, cytoplasmic proteins, such as the crystallins, are not able to maintain their normal relationship, thus creating the opacification.

The expression of the lacZ gene in the lens fibers of α3 knockout mice indicates the cellular regions that contain expression of the promotor for the α3 connexin gene in mice. A similar expression of the α3 connexin gene in the lens has

been seen in rat (Paul et al., J. Cell Biol., 115:1077-1089 (1991)), chick (Jiang et al., Develop. biol., 168:649-661 (1995)), and cow (Jarvis et al., Invest. Ophthalmol. & Vis. Sci., 34:613-620 (1993)). In addition, lacZ staining was detected in embryos in both dorsal and ventral regions of the tip of the limb buds, the dorsal root ganglion cells, and the lens epithelial cells. These results implicate α3 connexin as a functional component of the cells in these regions during development. LacZ staining was not detectable in the adult heart, even though α3 transcripts have been detected in RNA extracts from mouse (unpublished observation) and rat heart (Paul et al., supra, (1991)).

Morphological analysis of the lenses of α3 (-/-) mice indicate that this is not a typical osmotic-type cataract, such as the diabetes cataract, (Bond et al., Invest. Ophthal. & Vis. Sci., 37:1557-1565 (1996)) or that found in the congenital MIP mouse (Bassnett and Shiels, Nat. Genet., 12::212-215 (1996)), in which swelling of the lens fibers has been observed. Instead, cataracts in the mouse model of the present invention display morphological features comparable to those found in senile human cataracts (Al-Ghoul et al., Exp. Eye Res., 62:237-251 (1996)). As such, the present model and methods of use thereof is predictive of efficacy of therapeutic treatment of human cataractogenesis. Biochemical data indicate that the nuclear cataracts result from the light scattering of high-molecular-weight insoluble aggregates of lens proteins formed by disulfide crosslinking between proteins. These types of changes have been reported also to occur in the human nuclear cataracts (Al-Ghoul et al., id., (1996); Spector, In: The Ocular Lens, Structure, Function and Pathology. H. Maisel, Ed. New York, Marcel Dekker, Inc. pp. 4055-438 (1985)).

From a histological analysis and measurements of lens weight, the differentiation of the lens fiber cells and the early growth of the lens were not affected by the absence of α3 connexin in the mice. However, the early onset of a nuclear cataract in α3 (-/-) mice establishes that α3 connexin is essential for maintenance of lens transparency. The slight reduction in the weight of the lenses from the older α3 (-/-) mice was likely to be a consequence of the pathogenesis of the nuclear cataract. The cataractogenesis in α3 (-/-) mice is unlikely to be due to lacZ protein expression for several reasons: a) previous studies have shown no evidence of cataract formation when the *lacZ* gene is highly expressed in the lens (Goring et al., Science, 235:456-458 (1987)); b) lacZ proteins were preferentially detected in the lens cortex, where no cataracts were observed; c) lenses of α3 (+/-) mice also expressed lacZ proteins in the lens, but exhibited no cataracts up to the age of 8 months.

Connexin α8 has been reported to be cleaved at its C-terminus to generate a 35 kDa form in the rat lens (White et al., Mol. Biol. Cell, 3:711-720 (1992); Lin et al., Eur. J. Cell Biol., 73:141-149 (1997)). The proteolytic processing of α8 connexin is first detected in the lens nucleus of the mice at postnatal day 5 by immunocytochemical staining (Evans et al., Eur. J. Cell Biol., 60:243-249 (1993)). Thus, it is possible that the intact α8 connexin might be sufficient to compensate for the loss of α3 connexin proteins during early lens development and in the differentiating fibers in the lens cortex. However, the fact that α8 connexin did not compensate for the loss of α3 in the lens nucleus might be due to the proteolysis of α8 connexin in the nuclear region.

The dispersed 9-11 nm particles shown in the right panel of figure 5E are likely to represent gap junction particle

aggregates in the lens deep cortex and nucleus (Costello et al., Oophthalm. & Vis. Sci., 30:975-989 (1989)) and these particles were often mixed among many small islands of square-array particles, thought to represent MIP channels that are formed by MP26 (Zampighi et al., J. Cell Biol., 108:2255-22275 (1989)). Square arrays have also been observed in several types of congenital and transgenic mouse models (Lo and Kuck, Curr. Eye Res., 6:433-444 (1987); Dunia et al., J. Cell Biol., 132:701-716 (1996)). The formation of square arrays has been correlated with the degradation of MP26 to MP22 (Costello et al., supra, (1989)). In contrast, square arrays have been observed in MDR3 transgenic mouse lenses in the absence of proteolytic degradation of MP26 (Dunia et al., supra, (1996)). No significant changes in the RNA and protein levels of MP26 and MP22 (the cleaved form of MP 26) were detected in the α3 (-/-) lenses (unpublished observations). The appearance of the large areas of square-array particles likely resulted from the significant reorganization of the membrane structure in the α3 (-/-) lens nucleus. This data suggests that α3 connexin might also play a structural role in the organization of membrane protein and lipid.

Immunoblotting verified that the degradation of γ-crystallins was detected only in the lens nucleus of α3 (-/-) mice, where γ-crystallins are normally enriched (Siezen et al., J. Mol. Biol., 199:405-438 (1998)). Several congenital nuclear cataracts have been directly associated with the modification of one or more of the γ-crystallin genes. Activation of a γE-crystallin pseudogene (ιγE gene), which expressed a 6 kD N-terminal γE-crystallin fragment, has been suggested to be the cause of the hereditary Coppock-like cataract in human (Brakenhoff et al., Human Mol. Genet., 3:279-283 (1994)). Degraded γ-crystallins in α3 (-/-) mice

are sufficient to initiate and accelerate the aggregation of the lens proteins in the nucleus to generate nuclear cataracts.

The putative cleavage site between Asp73 and Ser74 for γ-crystallins suggested in the present invention is different from that determined for a 9 kDa water-soluble γD crystallin fragment in human lens (Srivastava and Srivastava, Exp. Eye Res., 62:593-604 (1996)). It has been reported that γ-crystallins are resistant to the calpain II protease (David and Shearer, Exp. Eye Res., 42:227-238 (1986)). Therefore the degradation of γ-crystallins as described in the present invention is caused by the action of another protease in the lens. Comparison of the Asp-Ser cleavage site of the γ-crystallin with known protease substrate specificities indicates that caspase proteases of the cysteine protease family (Nicholson et al., Nature, 376:37-43 (1995)) and granzyme B (Caputo et al., Nature Structural Biol., 1:364-367 (1994)) can cleave at an Asp-X site. Both proteases have been reported to play a critical role in apoptosis (Patel et al., supra, (1996)). Different members of the caspase family have been reported to be expressed in many different organs and cell types. The present invention thus describes a caspase-related protease is responsible for the cleavage of γ-crystallin. The action of this enzyme may play an important role in both maturation of lens fibers in normal lenses and cataractogenesis in α3 (-/-) lenses.

Apoptosis has been reported in the lens epithelial cells (Li et al., J. Cell Biol., 130:169-181 (1995)). The maturation of lens fiber cells to promote transparency includes complete degradation of all intracellular organelles including the nucleus, endoplasmic reticulum, and mitochondria. While this process resembles some features of

apoptosis (Chaudun et al., J. Cell Physiol., 158:354-364 (1994)), the molecular mechanisms involved in this process are poorly understood and are reported to differ from classical apoptosis (Bassnett and Mataic, J. Cell Biol., 137:37-49 (1997)).

The function of α-crystallin as a chaperone protein that prevents denaturation and aggregation of crystallins *in vitro* (Rao et al., Biochim. Biophys. Acta, 1245:439-447 (1995)) and *in vivo* (Brady et al., Proc. Natl. Acad. Sci., USA, 94:844-889(1997)) has been described. The profile of degraded α-crystallins in α3 (-/-) lenses was remarkably similar to that present within mature fibers normally. The putative cleavage site of αB crystallin *in vivo* can be reproduced *in vitro* using calpain II protease (Yoshida et al., Ophthal. & Vis. Sci., 27:1269-1273 (1986)). Degradation of the C-terminus of α-crystallins could reduce their chaperone function (Takemoto, Exp. Eye Res., 59:2399-242 (1994)). Calpain II has been reported to play a critical role in several cataract models (Shearer et al., Curr. Eye Res., 11:357-369 (1992); Mitton et al., J. Biol. Chem., 271:31803-31806 (1996); Truscott et al., Ophthal. & Vis. Sci., 31:2405-2411 (1990); Yoshida et al., Yoshida, Curr. Eye Res., 4:983-988 (1985). It is possible that calpain II may also be involved in the cataractogenesis of α3 (-/-) mice. However, based on the evidence that extensive degradation of αB crystallin was detected in the transparent cortex of the nuclear cataract lens from α3 (-/-) mice, the degradation of αB crystallin is not by itself sufficient to generate the opaque lens. Calpain II may be responsible for the degradation of αB- and β-crystallin, but not γ-crystallin. Further, no degradation of γ-crystallin has been reported in cataract models mediated by the activation of calpain II (Mitton et al., J. Biol. Chem., 2271:31803-311806

(1996); Shearer et al., Curr. Eye Res., 11:357-369 (1992); Truscott et al., supra, (1990); Yoshida et al., supra, (1985)). Thus, the observed degradation of γ-crystallin in α3 (-/-) lenses is not a consequence of the activation of calpain II. While ubiquitin has been found in macromolecular aggregates of crystallin (Jahngen-Hodge et al., Exp. Eye Res., 55:897-902 (1992)) its involvement in the generation of the cataract phenotype in the α3 (-/-) mouse lens remains to be clarified.

Cataract formation is often associated with changes in the ionic balance in the lens, especially the homeostasis of calcium ions, which have an important role in the activation of proteolytic enzymes (Duncan et al., Prog. In Retinal & Eye Res., 13:623-652 (1994)). Indeed, gap junction channels might be a major pathway for transporting calcium ions among the lens fiber cells.

In summary, the changes in the crystallins observed in the cataractogenesis of the α3 (-/-) mice suggest that gap junctional channels in the lens transport metabolic substrates (small molecules) that are ultimately important for the regulation of an unknown protease(s) and maintenance of the superstructural organization of lens proteins responsible for lens transparency. The morphological results in this study also suggest that α3 connexin might have a structural role in the organization of lens membrane components. Finally, the potential relevance of this mouse model for studying human cataracts is supported by the recent report that a human autosomal dominant cataract has been mapped to the same region on human chromosome 13 where the α3 connexin gene is located (Mackay et al., J. Cell Biol., 130:169-181 (1997)).

2. Assays to Screen for Inhibitors of Cataract Growth and of Interleukin-1β Converting Enzyme (ICE)

A. Cataract Growth

The present invention contemplates methods both *in vivo* and *in vitro* for identifying inhibitors of cataract growth, either prophlactically or therapeutically. Thus, the methods are useful in identifying a compound that can prevent cataract formation, alter the progression of cataract formation by delay or inhibiting the complete formation of a cataract or effecting a reversal or regression of a cataract at any stage of formation.

One exemplary method is the application of a selected compound to the α3 connexin-deficient mouse prepared in Example 1. Administration is effected topically or intravenously. The effect of the compound on growth of the cataract is then evaluated visually by observing a change in the amount of opacity as previously described in Example 1. Alternative ways to assess effects on cataract growth include the biological assays as described in Example 1 including morphological and biochemical analyses of lens isolated from the mouse having received compound applications.

The effect of a compound on a lens is also assessed through an *in vitro* system in which the lens of the mouse described above is cultured according to methods well known in the art for explant organ culture. The compound is directly applied to the medium in which the lens is cultured. Effects on opacification are assessed visually, morphologically or biochemically as previously described.

B. ICE Inhibitors

The present invention also describes methods to to identify an inhibitor of interleukin-1β converting enzyme

(ICE) or functional ICE analog. Inhibitors are identified with a variety of approaches. Two exemplary methods utilize the non-human mammal of the present invention and the discovery of the degradation pattern of the γ-crystallin lens protein as a result of the α3 connexin gene disruption in the non-human mammal.

In one method, a γ-crystallin lens protein is contacted with a solution of ICE or functional ICE analog to form a mixture in the presence of a candidate inhibitor of ICE or functional ICE analog under conditions described in Example 1 for assessing cleavage patterns of γ-crystallin. The effect of the inhibitor is then assessed by comparing the cleavage patterns of untreated and treated samples. An effective inhibitor is identified with the presence of an altered cleavage pattern compared to that of undegraded γ-crystallin.

For these assays, the lens protein is in either purified or unpurified form. The ICE or functional ICE analog is either commericially obtained or is providedin a homogenate of lens isolated from the non-human mammal as described in Example 1.

Another screening method to identify an inhibitor of ICE or a functional ICE analog is based on the use of an ICE or functional ICE homolog substrate well known to those of skill in the art of apoptosis. Exemplary ICE substrates include γ-crystallin and precursor interleukin-1β (pIL-1β) and are described in US Patents 5,607,831 and 5,656,627, the disclosures of which are hereby incorporated by reference. The substrate is mixed with a homogenate of lens isolated from the non-human mammal prepared in Example 1. The homogenate contains ICE or functional ICE analog as described in Example 1 that results in the novel cleaveage pattern of γ-crystallin. The above mixture is prepared in the presence of a candidate

inhibitor of ICE or functional ICE analog under conditions where ICE or functional ICE analog is known to cleave the substrate generating detectable cleavage products. The cleavage pattern obtained with the test compound is compared to that with a control thereby identifying the effectiveness of the test compound as an inhibitor of the novel protease in the lens.

3. Application of Inhibitors of Interleukin-1β Converting Enzyme (ICE) to a Subject to Prevent or Treat Cataracts

The present invention contemplates methods of prophylatic or therapeutic treatment of cataract growth in a subject. Based on the present discovery of the unique γ-crystallin cleavage pattern in α3 connexin-deficient mammals and effects on cataractogenesis as discussed in Example 1, a prophylactically or therapeutically effective cataract growth affecting amount of an interleukin-1β converting enzyme (ICE) inhibitor administered to a subject patient. ICE inhibitors include but are not limited to the following molecules: Bcl2, Bcl-XL, CrmA (cowpox virus protein), ZVAD-fluromethylketone (a permeant ICE inhibitor), lactacystin, dichloroisocourmarin, peptides Ac-YVAD-CMK and CPP32/Yama, and DEVD-CHO, and those described in US Patents 5,656,627, 5,552,536, 5,672,500 and 5,654,146, the disclosures of which are hereby incorporated by reference.

For treament, the candidate inhibitor is administered orally, intravenously, topically, or the like routes. For topical administration to the eye, the compound is preferably providied in association with an opthalmalogically acceptable carrier. Preferred opthamalogical modalities are provided in US Patents 5,401,880, 5,422,376, 5,519,054, 5,578,578 and 5,628,801, the disclosures of which are hereby incorporated by

reference.

The effect of the inhibitor is evaluated most easily by direct examination of the lens optometrically.

4. Deposit of Materials

The following plasmid has been deposited on or before January 10, 1997, with the American Type Culture Collection, 1301 Parklawn Drive, Rockville, MD, USA (ATCC):

Material	ATCC Accession No.
plasmid pα3-GZK	97848

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable plasmid for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty which assures permanent and unrestricted availability of the progeny of the plasmid to the public upon issuance of the pertinent US patent or upon laying open to the public of any US or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the US Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the plasmid deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same type. Availability of the deposited plasmid is not to be

construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the plasmid deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any transgenic mammals produced thereby that are functionally equivalent are within the scope of this invention. The deposit of material does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What Is Claimed Is:

1. An isolated nucleic acid sequence defining a disrupted α3 connexin gene.

2. The nucleic acid sequence of claim 1 wherein the disrupted α3 connexin gene lacks at least one transmembrane-encoding domain.

3. The nucleic acid sequence of claim 1 wherein the disrupted α3 connexin gene lacks four transmembrane-encoding domains.

4. A targeting vector comprising a nucleic acid sequence defining a disrupted α3 connexin gene.

5. The targeting vector of claim 4 wherein the wherein the disrupted α3 connexin gene lacks at least one transmembrane-encoding domain.

6. The targeting vector of claim 4 wherein the wherein the disrupted α3 connexin gene lacks four transmembrane-encoding domains.

7. The targeting vector of claim 6 comprising a plasmid having the ATCC Accession Number 97848.

8. The targeting vector of claim 4 further comprising a selectable marker.

9. The targeting vector of claim 8 wherein the selectable marker is a neomycin gene.

10. The targeting vector of claim 4 further comprising a marker sequence.

11. The targeting vector of claim 10 wherein the marker sequence is a *lacZ-nls* reporter gene.

12. A mutant non-human mammal wherein its endogenous α3 connexin gene has been disrupted by homologous recombination using an exogenous targeting vector comprising a disrupted α3 connexin gene.

13. The mammal of claim 12 wherein the disrupted α3 connexin gene lacks at least one transmembrane-encoding domain.

14. The mammal of claim 12 wherein the wherein the disrupted α3 connexin gene lacks four transmembrane-encoding domains.

15. The mammal of claim 14 wherein the transmembrane-encoding domains are replaced with a *lacZ-nls* reporter gene.

16. The mammal of claim 15 wherein the targeting vector comprises a plasmid having the ATCC Accession Number 97848.

17. The mammal of claim 16 wherein the mammal is a mouse.

18. The mammal of claim 12 wherein the disruption prevents the expression of functional α3 connexin protein.

19. The mammal of claim 12 wherein the disruption prevents the expression of α3 connexin protein.

20. The mammal of claim 12 wherein the disruption is introduced into the mammal or an ancestor of the mammal at an embryonic stage, and wherein the disruption results in the development of a cataract in an α3 homozygous (-/-) mammal.

21. A method for producing a non-human mammal containing a disrupted α3 connexin gene, the method comprising:

a) integrating, via homologous recombination, the targeting vector of claim 4 into a non-human mammalian α3 target gene present in an embryonic stem cell to form a recombined non-human mammalian disrupted α3 target gene in an allele in the stem cell;

b) selecting a stem cell containing the disrupted α3 gene in an allele;

c) producing a viable non-human mammal containing a disrupted α3 gene in an allele.

22. The method of claim 21 wherein the non-human mammal

is an α3 homozygous (-/-) mammal.

23. A method to identify a compound for use in affecting growth of a cataract, the method comprising:

a) administering the compound to a lens of a non-human mammal of claim 12, and

b) evaluating the effect of the compound on growth of the cataract.

24. The method of claim 23 wherein the compound is topically applied to an eye of the non-human mammal.

25. The method of claim 24 wherein the topical administration is in association with an opthalmalogically acceptable carrier.

26. The method of claim 22 wherein the compound is administered in association with an acceptable pharmaceutical carrier.

27. The method of claim 26 wherein the compound is administered intravenously to the non-human mammal.

28. The method of claim 22 wherein the non-human mammal is an α3 heterozygous (+/-) mammal.

29. The method of claim 22 wherein the lens is cultured *in vitro*.

30. The method of claim 29 wherein the compound is administered to the cultured lens.

31. The method of claim 22 wherein the affected growth is formation of a cataract.

32. The method of claim 31 wherein the compound is administered prior to the formation of a cataract.

33. The method of claim 22 wherein the affected growth is preventing progression of cataract formation.

34. The method of claim 33 wherein the compound is administered to the lens having a detectable cataract.

35. The method of claim 22 wherein the affected growth

is reversing cataract formation.

36. The method of claim 35 wherein the compound is administered to the lens having a detectable cataract.

37. An article of manufacture comprising a packaging material and contained therein in a separate container the targeting vector of claim 7, and wherein the packaging material comprises a label which indicates that the targeting vector can be used to disrupt an α3 gene in a non-human mammal.

38. A method of affecting cataract growth in a subject, the method comprising administering to the subject a therapeutically effective cataract growth affecting amount of an interleukin-1β converting enzyme (ICE) inhibitor.

39. The method of claim 38 wherein the inhibitor is selected from the group consisting of Bcl2, BCL-XL, CrmA, lactacystin, and dichloroisocourmarin.

40. The method of claim 38 wherein the inhibitor is administered orally.

41. The method of claim 38 wherein the inhibitor is administered intravenously.

42. The method of claim 38 wherein the inhibitor is administered topically.

43. The method of claim 42 wherein topical administration is on an eye.

44. The method of claim 42 wherein topical administration is in association with an opthalmalogically acceptable carrier.

45. The method of claim 38 wherein the inhibitor is administered in association with an acceptable pharmaceutical carrier.

46. The method of claim 38 wherein the inhibitor prevents cataract formation.

47. The method of claim 38 wherein the inhibitor delays progression of cataract formation.

48. The method of claim 38 wherein the inhibitor stops progression of cataract formation.

49. The method of claim 38 wherein the inhibitor causes regression of a formed cataract.

50. A screening method to identify an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog, the method comprising the steps of:

a) contacting a γ-crystallin lens protein with ICE or functional ICE analog in the presence of a candidate inhibitor of ICE or functional ICE analog under conditions wherein ICE or functional ICE analog is known to cleave the lens protein generating detectable cleavage products; and

b) detecting an altered cleavage pattern, thereby identifying the inhibitor.

51. The method of claim 50 wherein the lens protein is purified.

52. The method of claim 50 wherein ICE or functional ICE analog is present in a homogenate of lens isolated from the non-human mammal of claim 12.

53. The method of claim 50 wherein the lens protein, ICE or functional ICE analog, and the candidate inhibitor are in solution.

54. A screening method to identify an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog, the method comprising the steps of:

a) contacting a substrate of ICE or functional ICE homolog with a homogenate of lens isolated from the non-human mammal of claim 12, wherein the homogenate contains ICE or functional ICE analog, in the presence of a candidate inhibitor of ICE or functional ICE analog under conditions

wherein ICE or functional ICE analog is known to cleave the substrate generating detectable cleavage products; and

b) detecting an altered cleavage, thereby identifying the inhibitor.

55. The method of claim 54 wherein the substrate, lens homogenate, and the candidate inhibitor are in solution.

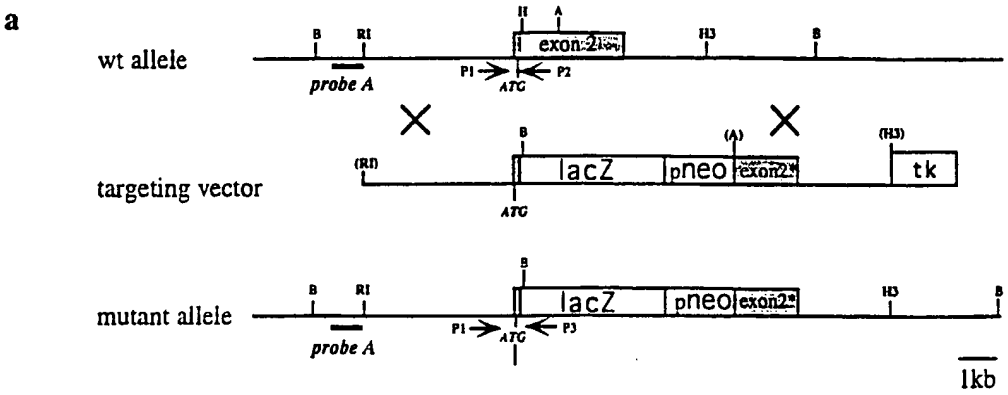
56. A kit for identifying an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog, the kit comprising packaging means comprising a first container containing a γ-crystallin lens protein in an amount sufficient for at least one assay, and comprising a packaging material comprising a label indicating that the crystallin lens protein can be used to identify the inhibitor by detection of an altered cleavage pattern.

57. The kit according to claim 56 further comprising a second container containing ICE or functional ICE analog.

58. The kit of claim 57 wherein ICE or functional ICE analog is present in a homogenate of lens isolated from a non-human mammal of claim 12.

59. A kit for identifying an inhibitor of interleukin-1β converting enzyme (ICE) or functional ICE analog, the kit comprising packaging means comprising a first container containing a substrate of ICE or functional homolog and a second container comprising a homogenate of lens isolated from a non-human mammal having disrupted alleles of α3 connexin gene; and comprising a packaging material comprising a label indicating that the crystallin lens protein can be used to identify the inhibitor by detection of an altered cleavage pattern.

60. The kit of claim 59 wherein the substrate is a γ-crystallin lens protein.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wt allele
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P2
ATG
exon 2
targeting vector
lacZ
pneo
exon2*
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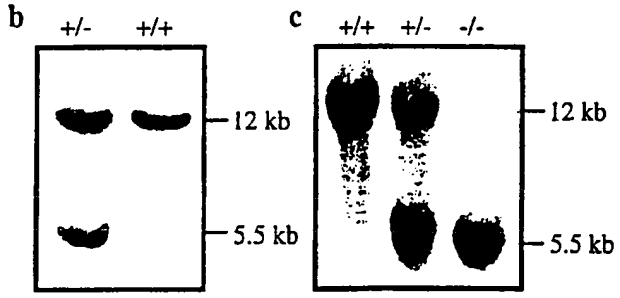
mutant allele
P3
1kb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+/-
+/+
12 kb
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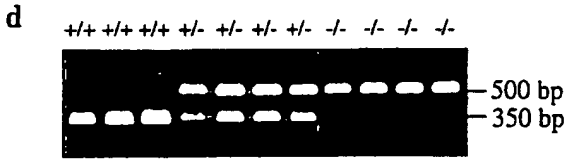
c
+/+
+/-
-/-
12 kb
5.5 kb


d
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500 bp
350 bp

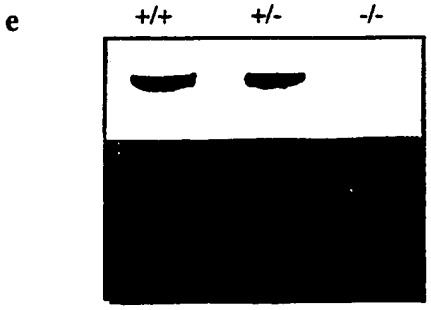

e
+/+
+/-
-/-

FIGURE 1

FIGURE 2

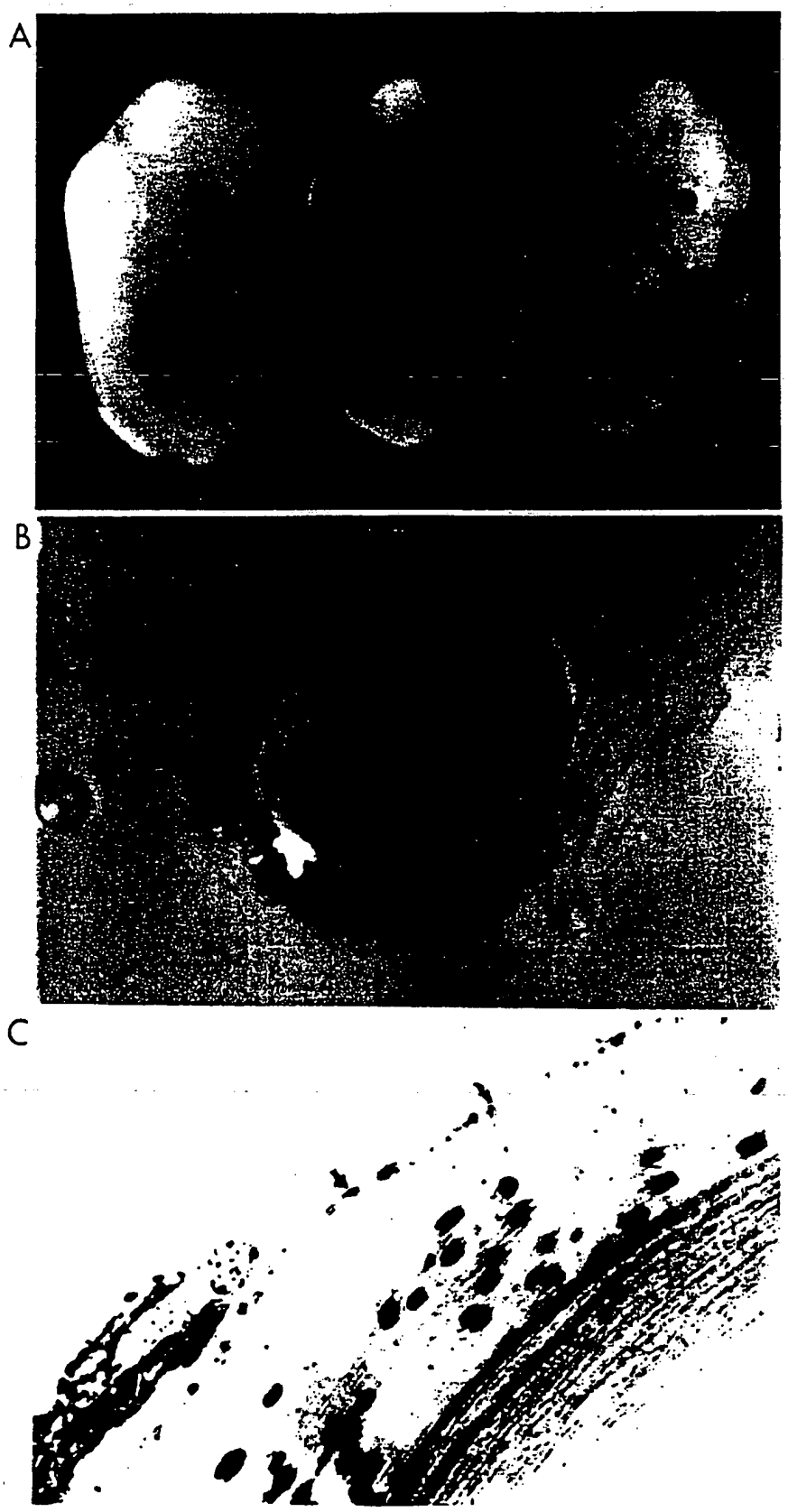
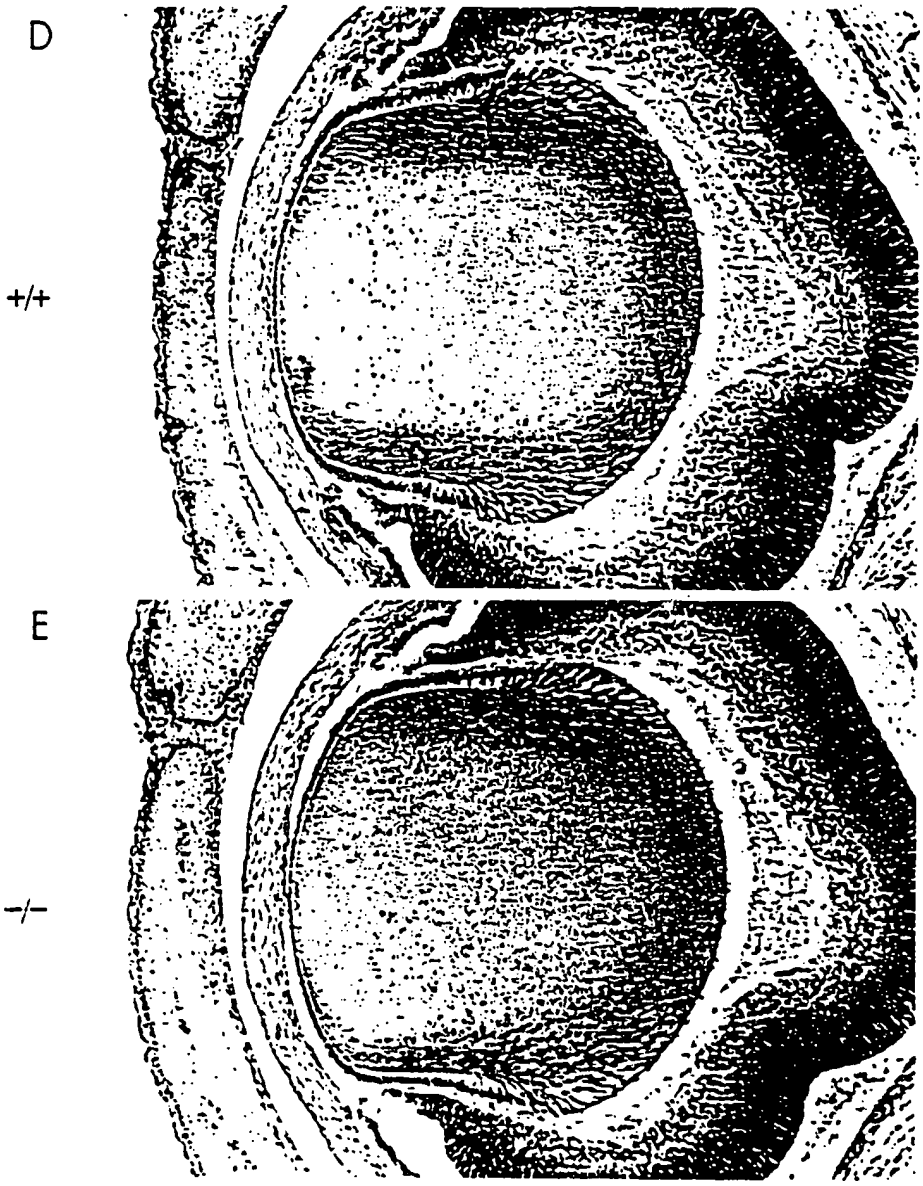


FIGURE 2



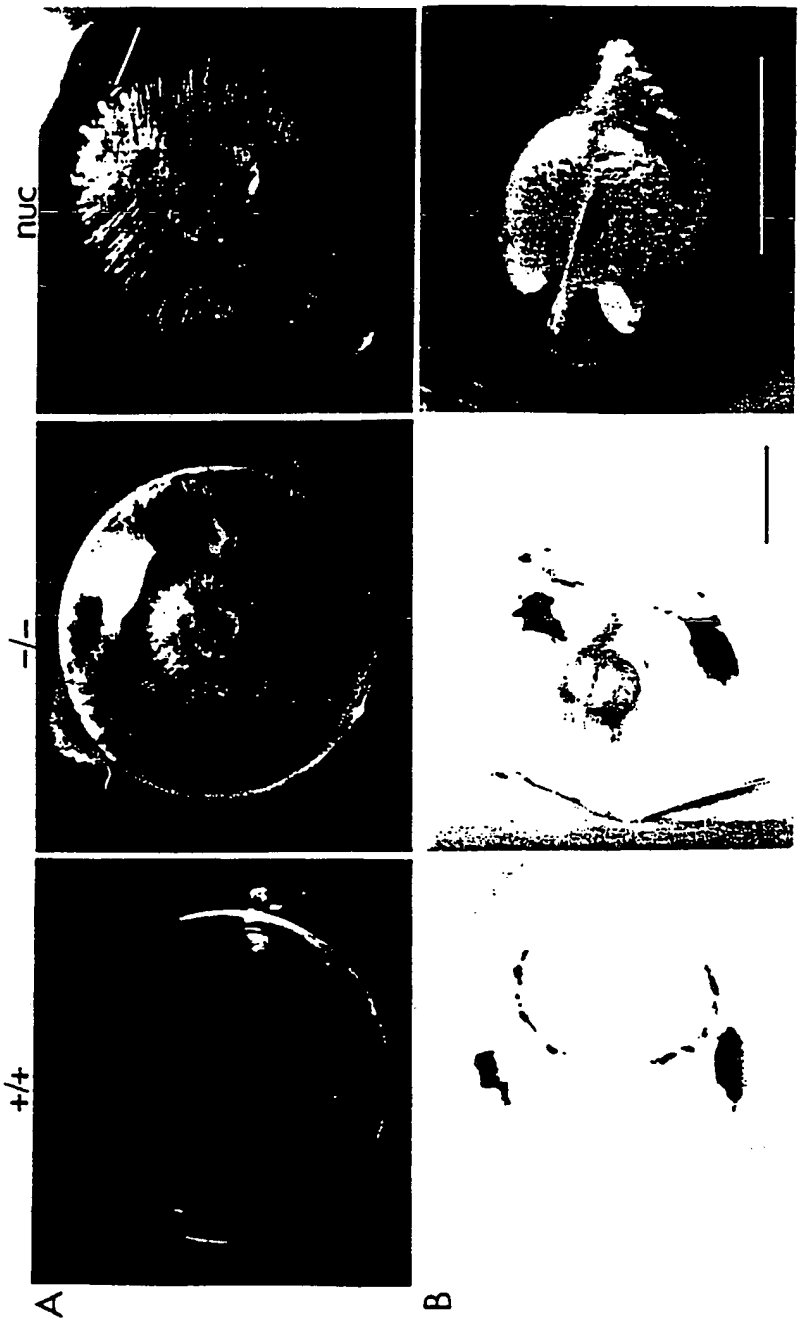


FIGURE 3

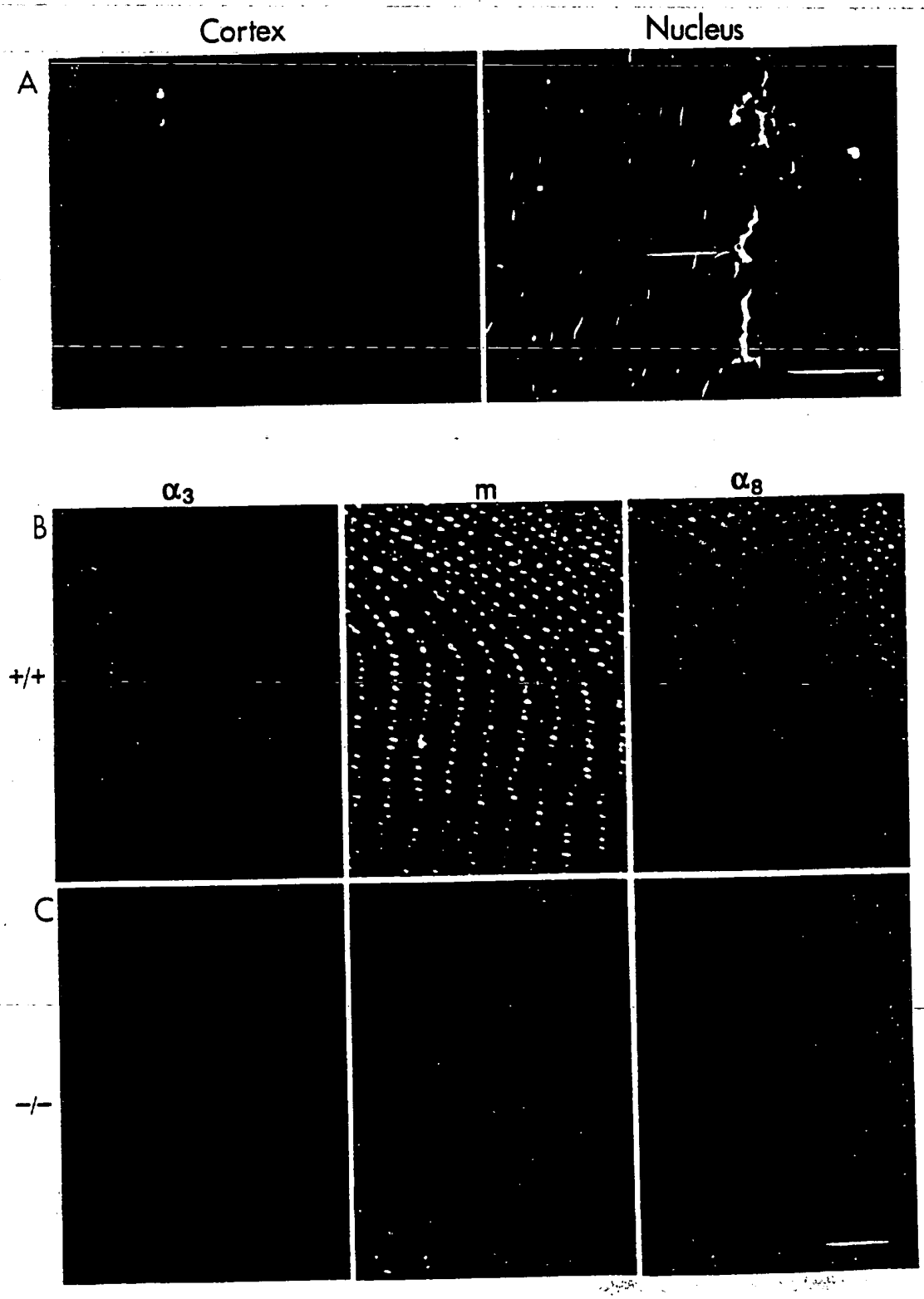
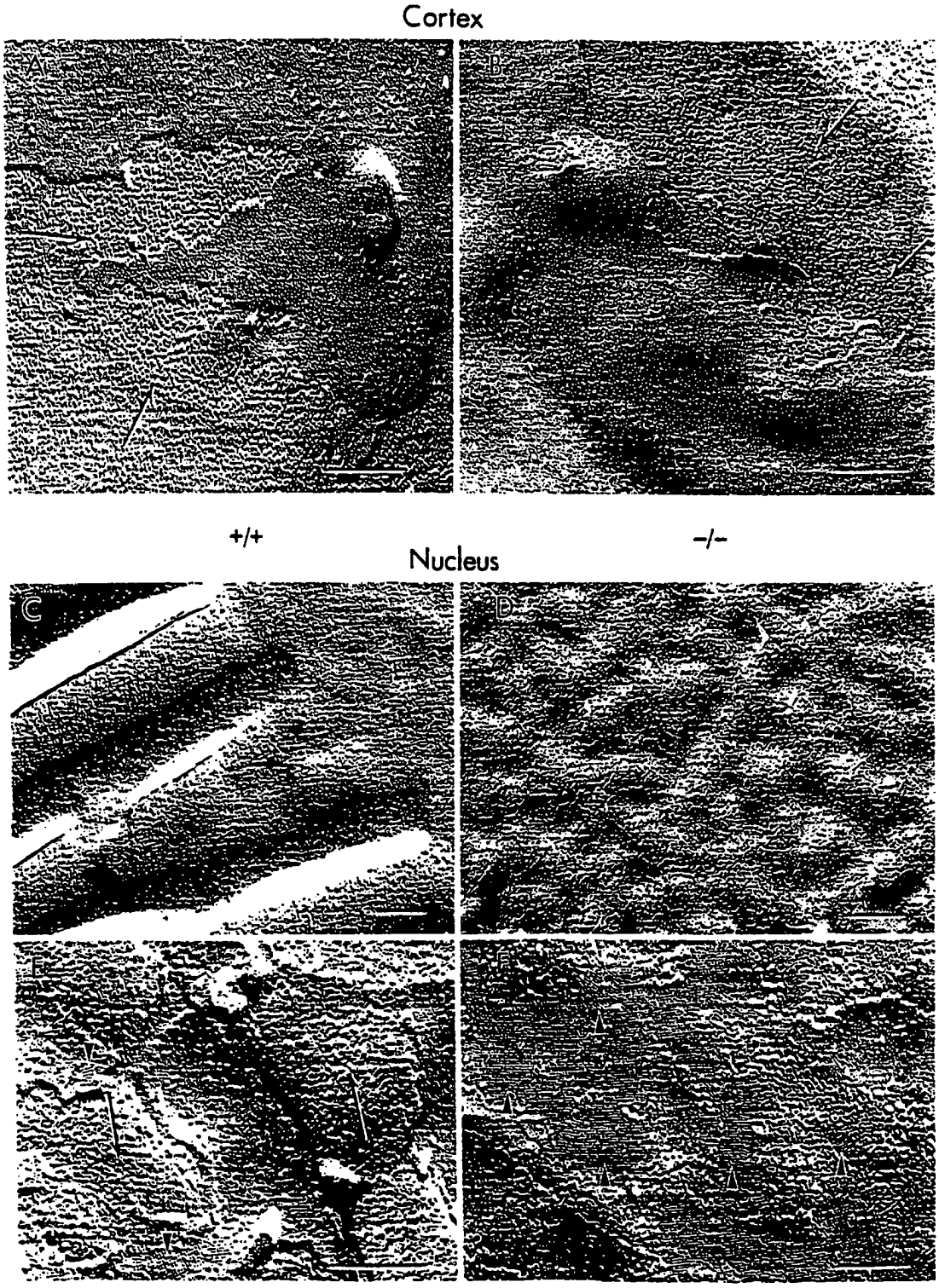


FIGURE 4

FIGURE 5



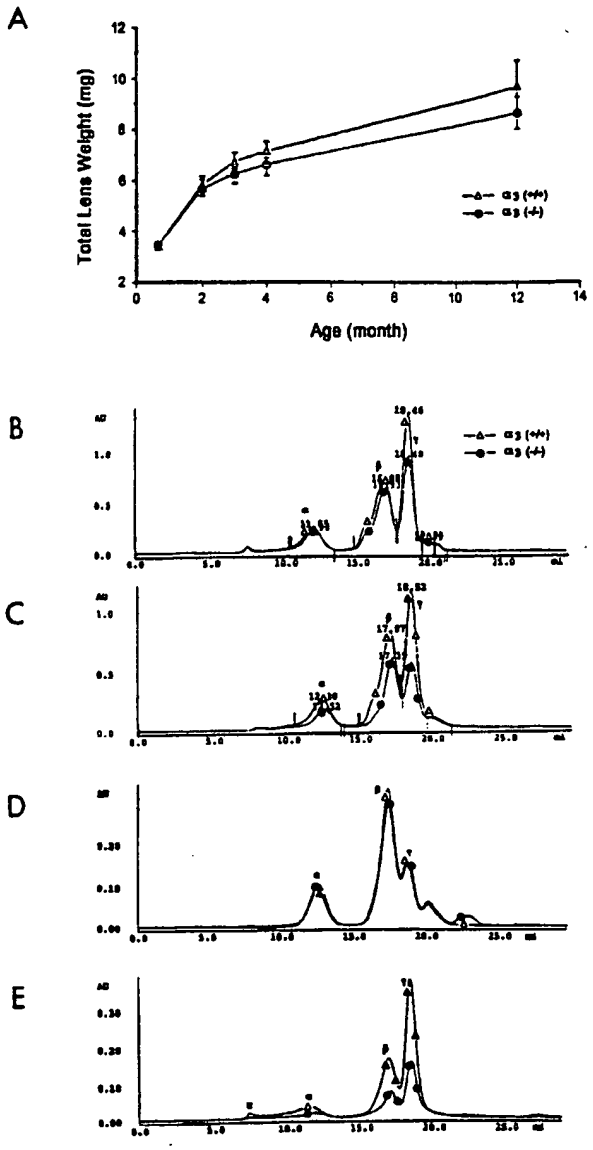


FIGURE 6

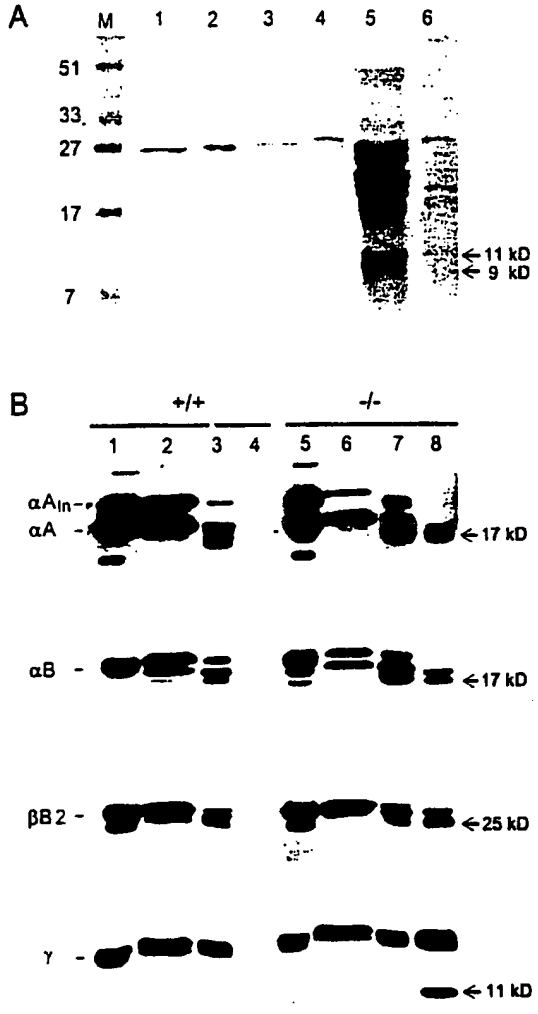

A
M
1
2
3
4
5
6
51
33
27
17
7
11 kD
9 kD
B
+/+
-/-
1
2
3
4
5
6
7
8
αA
αA
17 kD
αB
17 kD
βB2
25 kD
γ
11 kD

FIGURE 7

FIGURE 8A

```
ATG GGC GAC TGG AGC TTC CTG GGG CGG CTG CTG GAG AAC GCA CAG GAG        48
Met Gly Asp Trp Ser Phe Leu Gly Arg Leu Leu Glu Asn Ala Gln Glu
  1               5                  10                  15

CAC TCT ACA GTC ATC GGC AAA GTG TGG CTG ACC GTG CTG TTC ATC TTC        96
His Ser Thr Val Ile Gly Lys Val Trp Leu Thr Val Leu Phe Ile Phe
             20                  25                  30

CGC ATT CTG GTG TTA GGG GCG GCA GCC GAG GAG GTG TGG GGC GAC GAG       144
Arg Ile Leu Val Leu Gly Ala Ala Ala Glu Glu Val Trp Gly Asp Glu
         35                  40                  45

CAA TCG GAC TTC ACC TGC AAC ACA CAG CAG CCA GGC TGT GAG AAC GTC       192
Gln Ser Asp Phe Thr Cys Asn Thr Gln Gln Pro Gly Cys Glu Asn Val
     50                  55                  60

TGC TAC GAC CGC GCT TTC CCC ATT TCG CAC ATC CGC TTC TGG GCG CTG       240
Cys Tyr Asp Arg Ala Phe Pro Ile Ser His Ile Arg Phe Trp Ala Leu
 65                  70                  75                  80

CAA ATC ATC TTC GTG TCT ACG CCC ACC CTC ATC TAT CTG GGC CAC GTG       288
Gln Ile Ile Phe Val Ser Thr Pro Thr Leu Ile Tyr Leu Gly His Val
                 85                  90                  95

CTA CAC ATC GTG CGC ATG GAG GAG AAG AAG AAA GAG CGG GAG GAA GAG       336
Leu His Ile Val Arg Met Glu Glu Lys Lys Lys Glu Arg Glu Glu Glu
            100                 105                 110

CTG CTG AGG AGA GAC AAC CCT CAG CAC GGC CGT GGT CGC GAG CCA ATG       384
Leu Leu Arg Arg Asp Asn Pro Gln His Gly Arg Gly Arg Glu Pro Met
        115                 120                 125

CGT ACA GGG AGC CCG CGG GAC CCT CCA CTA CGC GAT GAC CGT GGC AAG       432
Arg Thr Gly Ser Pro Arg Asp Pro Pro Leu Arg Asp Asp Arg Gly Lys
    130                 135                 140

GTG CGC ATC GCA GGT GCG CTG CTG CGG ACC TAC GTC TTC AAC ATC ATC       480
Val Arg Ile Ala Gly Ala Leu Leu Arg Thr Tyr Val Phe Asn Ile Ile
145                 150                 155                 160

TTC AAG ACA CTC TTC GAA GTG GGG TTC ATC GCG GGC CAG TAC TTT CTA       528
Phe Lys Thr Leu Phe Glu Val Gly Phe Ile Ala Gly Gln Tyr Phe Leu
                165                 170                 175

TAC GGC TTC CAG CTG CAG CCA CTT TAC CGC TGC GAC CGC TGG CCC TGC       576
Tyr Gly Phe Gln Leu Gln Pro Leu Tyr Arg Cys Asp Arg Trp Pro Cys
            180                 185                 190
```

FIGURE 8B

```
CCC AAC ACT GTG GAC TGT TTC ATC TCC AGG CCC ACA GAG AAG ACC ATC        624
Pro Asn Thr Val Asp Cys Phe Ile Ser Arg Pro Thr Glu Lys Thr Ile
        195                 200                 205

TTT GTC ATC TTC ATG CTG GCT GTG GCC TGT GCG TCA CTG GTA CTC AAC        672
Phe Val Ile Phe Met Leu Ala Val Ala Cys Ala Ser Leu Val Leu Asn
    210                 215                 220

ATG CTG GAG ATT TAC CAC CTG GGC TGG AAG AAG CTC AAG CAG GGA GTT        720
Met Leu Glu Ile Tyr His Leu Gly Trp Lys Lys Leu Lys Gln Gly Val
225                 230                 235                 240

ACT AAC CAC TTC AAC CCA GAT GCC TCA GAA GCC AGG CAC AAG CCC TTG        768
Thr Asn His Phe Asn Pro Asp Ala Ser Glu Ala Arg His Lys Pro Leu
                245                 250                 255

GAC CCC CTA CCC GCG GCC ACC AGC TCT GGC CCA CCC AGC GTC TCC ATC        816
Asp Pro Leu Pro Ala Ala Thr Ser Ser Gly Pro Pro Ser Val Ser Ile
            260                 265                 270

GGG TTC CCA CCT TAT TAC ACA CAC CCT GCC TGT CCC ACA GTA CAG GGA        864
Gly Phe Pro Pro Tyr Tyr Thr His Pro Ala Cys Pro Thr Val Gln Gly
        275                 280                 285

AAG GCC ATA GGG TTT CCT GGG GCC CCA CTA TCA CCA GCA GAC TTC ACA        912
Lys Ala Ile Gly Phe Pro Gly Ala Pro Leu Ser Pro Ala Asp Phe Thr
    290                 295                 300

GTG GTG ACT CTA AAC GAT GCC CAA GGC AGA AAC CAC CCA GTC AAA CAC        960
Val Val Thr Leu Asn Asp Ala Gln Gly Arg Asn His Pro Val Lys His
305                 310                 315                 320

TGC AAT GGC CAC CAC CTG ACG ACA GAG CAG AAC TGG ACC AGG CAA GTG       1008
Cys Asn Gly His His Leu Thr Thr Glu Gln Asn Trp Thr Arg Gln Val
                325                 330                 335

GCA GAG CAG CAG ACT CCA GCC AGC AAG CCC TCT TCA GCA GCA TCC AGC       1056
Ala Glu Gln Gln Thr Pro Ala Ser Lys Pro Ser Ser Ala Ala Ser Ser
            340                 345                 350

CCT GAT GGC CGC AAG GGG CTC ATT GAC AGC AGT GGC AGC AGC TTA CAG       1104
Pro Asp Gly Arg Lys Gly Leu Ile Asp Ser Ser Gly Ser Ser Leu Gln
        355                 360                 365

GAG AGT GCC TTG GTA GTG ACG CCA GAG GAG GGG GAA CAG GCT TTG GCC       1152
Glu Ser Ala Leu Val Val Thr Pro Glu Glu Gly Glu Gln Ala Leu Ala
    370                 375                 380

ACC ACA GTG GAG ATG TAC TCG CCA CCG TTG GTC CTC CTG GAC CCA GGA       1200
Thr Thr Val Glu Met Tyr Ser Pro Pro Leu Val Leu Leu Asp Pro Gly
385                 390                 395                 400
```

FIGURE 8C

```
AGG TCC AGC AAG TCC AGT AAC GGA CGT GCC AGA CCA GGT GAC TTG GCC      1248
Arg Ser Ser Lys Ser Ser Asn Gly Arg Ala Arg Pro Gly Asp Leu Ala
                405                 410                 415

ATC TAGCCATAGT CATTCCAGAT ACCTTTATAA ACCTTCTCTT TACCCCTTAG          1301
Ile

AAACCAAAAC ACTCAAAAAG CATTTCTAAT CAACAGGTAG AAAGAGAGAG AGAGGAAAAA   1361

ACCAAAACCA AAAAACCTGA CAGTGCTGGA TTCCCCTGAG GGAAACACTG AGCAAACTGG   1421

ATCAGTCTGG TTTTAATGCT TGCTGTTCTT GTCCCTGTGG GACAGGGTGG GGGTGACCCC   1481

AGTGTCTGAG ATCGAGGTGG GGGCAGAGCA AGGTTGTTGG CTCCCAGCAG TGCTTGCTCT   1541

TGTTCGGCCT CCTCCACTAT TCTGTCATGA TGAAGCAACA GGTGTTANCA GCCCTTCAGT   1601

GGGGGGACAA NTCAGTTTCC ATTCCTAAGC TCTCCAAGCA GCCCAGAGAG TCACAGAGGC   1661

ACTTAAGCTC TATCTCTGCT TGAGACTTTT AACTTAGAGT TCAATTTCAT TTTGGGATAT   1721

TTGCCAAACT GTACTAAAAA AAGACTTGGA ATCTATCTAG TGTCGCTTGA GCTTCTGGAA   1781

TTGCATGTGA GGTCCAGGAC ATCCAACTGC AGGCTGCAGG ACNCAGCGAC AGAGTAACAG   1841

TTGTCCTCCA GTGCTGTTTC CATGGGACAA GGCCACAGGC ATTCCTGGTA GAGGCAGGTG   1901

GAGATGCAGA GACCCTGGAA AGCCCTTCAA CAGAGGAGGA AGATGGTGGT GTGGCAGGTA   1961

TTGCCGTAGG TGTTCATTTA TTTCTTTGAT GATATTTTGT GCTTAGCCTG GATGGTGAAA   2021

GTATTTACCC CCCTCCCCCA CATACACATA TTTTGCCTTT CATTAAAAAA AATAGTGCCA   2081

TTAAAATGAC TACTGATCAG GGAGATGTTT AATTAGTTAA GATTTGGACT TTCCTTTAAA   2141

ACAAATCCAC CTTCCATGTC AGCCAACTAA TCACTATGAG GATGTCTAGG TAAAAAATTA   2201

ACCACTGTGA GAATGTACGC TAGATAAAAC TTGGGGTAGG GGATTCACAT GCGTTTTAAG   2261

TACTTGAAGT TTTAAAAGTT TCTGAATCGG AGGTTCACTA CTGTTACAAG GAAAAATGTT   2321

TATCCAGATG AATCATTGAT GACTCACACT TAAAAGAAAG TACACACTTA AAACACTTCA   2381

AG                                                                  2383
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00340

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00, 15/09, 15/63
US CL :800/2; 435/172.3, 69.1, 325, 320.1; 424/9.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/172.3, 69.1, 325, 320.1; 424/9.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PAUL et al. Connexin46, a Novel Gap Junction Protein, Induces Voltage-Gated Currents in Nonjunctional Plasma Membrane of *Xenopus* Oocytes. Journal of Cell Biology. November 1991, Vol. 115, No. 4, pages 1077-1089, see entire document.	1-22
Y	NELLES et al. Defective Propagation of Signals Generated by Sympathetic Nerve Stimulation in the Liver of Connexin32-Deficient Mice. Proceedings of the National Academy of Sciences of the United States of America. September 1996, Vol. 93, pages 9565-9570, see entire document.	1-60

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 APRIL 1998	Date of mailing of the international search report 26 MAY 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL D. SCHMUCK Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00340

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	REAUME et al. Cardiac Malformation in Neonatal Mice Lacking Connexin43. Science. 24 March 1995, Vol. 267, pages 1831-1834, see entire document.	1-60
A	BRUZZONE et al. Connections with Connexins: The Molecular Basis of Direct Intercellular Signaling. European Journal of Biochemistry. 1996, Vol. 238, pages 1-27, see entire document.	1-60
X --- Y	GONG et al. Disruption of Alpha-3 Connexin Gene Leads to Age-Related Cataract Formation in Mice. Molecular Biology of the Cell. 07-11 December 1996, Suppl. 7, page 509A, Abstract # 2961, see entire Abstract.	12-15, 17-22 --- 1-11, 16, 23-60
Y	CAPECCHI. Targeted Gene Replacement. Scientific American. March 1994, Vol. 270, No. 3, pages 34-41, see entire document.	1-60
Y	WESTPHAL. Transgenic Mammals and Biotechnology. FASEB Journal. 1989, Vol. 3, pages 117-120, see pages 119-120.	1-60
X, P ----- Y, P	GONG et al. Disruption of α_3 Connexin Gene Leads to Proteolysis and Cataractogenesis in Mice. Cell. 12 December 1997, Vol. 91, pages 833-843, see entire document.	1-6, 8-15, 17-22 --- 7, 16, 23-60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00340

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, GENBANK, BIOSIS, EMBASE, CAPLUS, WPIDS, USPATFULL, APS
search terms: connexin, alpha3 connexin, connexin46, transgene, homologous recombination, knockout, disrupt, mouse or mice, cataracts or crystalline lens, interleukin-1-beta converting enzyme, inhibit or cleave